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I am submitting herewith a dissertation written by Patsy Kylene Williams-Boyce entitled "Mammalian Ear Tissue Regeneration." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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MAMMALIAN EAR TISSUE REGENERATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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DEDICATION

This work is dedicated to Phyllis Bice, a most extraordinary woman whose professional and personal talents have been sources of inspiration and encouragement to me throughout my graduate career.

She is the framework of the Zoology Department. Her dedication to her job and the people around her show the pride she takes in her work. As a single parent, she manages to put in days and frequently evenings at UT and her freetime with the high school band, at ball games, and transporting cars full of children to lectures, plays, and speaking contests. She never stops giving to others; rarely do I see her take a moment for herself. In an age when most people are so caught up in the 'I or me' her unselfishness is truly refreshing. She is extremely motivated and capable at any task she undertakes from replacing her roof or building an elevated den, to getting a grant through administration. She never stops to say, "Can I do this", she just does it!

Days when I was tired or weary, a visit with Phyllis was adequate inspiration to go dig in again. A graduate student could have no better friend. She has run interference for me throughout of my graduate education. My friendship with her has been one of the pure pleasures of my graduate career. This dedicaton is but a small token of my friendship, love, and admiraton for her. Phyllis, thanks for always being there.

This dedication even though written months ago, seems now grossly inadequate as a means of thanking her. There could be no thanks of sufficient magnitude to adequately express my gratitude to her for sacrificing her time, sleep, and hours with her children to enable me to graduate. This dissertation was completed through her kindness, perseverance and hard work.

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ABSTRACT

Holes punched in the ears of rabbits are repaired by regeneration of new tissues from blastemas and the unwounded tissues found at the periphery of the wounds. The biochemical, physiological, morphological and kinetic parameters of this regeneration have been investigated both to characterize the system and to show how the process is similar to embryonic development. The proportion of animals which successfully regenerated was greater in males than females, and in ovariectomized does given testosterone than in those given only corn oil carrier; yet the speed of closure did not differ in these groups. Holes in juveniles closed faster than holes in older animals. Second holes punched on the same site where earlier tissues had been regenerated closed earlier, had a thicker cartilage layer, and a higher success rate than primary punches indicating a 'primary' effect from the primary punch. Holes punched closer to the head were more successful than those punched distally and holes punched on the edge of the pinna did not regenerate. Regenerated hair reestablished the original color pattern, and the hair regenerating after punching through transplants of back skin to the ear had characteristics of back skin hair in color, length, and orientation.

Other mammals were found capable of partial ear tissue regeneration when punches were made on the proximal pinna. Similar morphological characteristics were shared among the successful animals.

Biochemical changes in collagen were monitored throughout the regeneration process and these changes were similar to those seen in the embryonic development of cartilage containing tissues. Type I collagen was found in skin and connective tissue and Type II in cartilage at day 0. Type II was not

found during early regeneration but Type I and two unusual types, Type I-Trimer, and Type III were found. Type II was found again once chondrogenesis began and the two unusual types were no longer found. Amounts of soluble collagen varied during the process and collagenase was inversely correlated with the changes in collagen.

Similarities between regenerating tissues and embryonic supporting tissues were observed: Gallium-67 was found to localize in regenerating ear tissues, and a protein was found which cross-reacted with antibodies to both the high molecular weight and uteroglobin fractions of rabbit uterine flushings taken on day 5 post coitum. It is suggested that regenerating tissues exhibit a greater similarity to embryonic tissues than to tissues comprising a wound.

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CHAPTER I

INTRODUCTION

Regeneration as a Biological Process

The primary aim of this study is to survey various parameters of the rabbit ear tissue regeneration system and ultimately to compare a number of these parameters with embryonic processes. Regeneration remains one of the intriguing enigmas of developmental biology and the events of regeneration raise a number of important questions. The most common of these is why the tailed amphibia can regenerate limbs whereas mammals cannot. These studies on regeneration in a mammal may offer information leading to the answers to other questions such as: How this developmental process occurring in the adult compare with embryonic development, defining certain restrictions of regeneration, and how biochemical changes occurring within the tissues might provide pathways for restoration of the lost tissues.

Regeneration, taken from the Latin, means to produce again, i.e. the reestablishment of lost form and function. The initial stimulus (requirement) for most regeneration events is usually an injury, amputation, or loss of a structure. The second requirement is that some part of the original structure must be left behind to serve as a source of building materials, i.e. regeneration can replace a part but never a whole. Protozoa need the nucleus to regenerate, flatworms need the brain for eye regeneration, newt lens needs the retina to regenerate and newt limbs require a stump to regenerate.

Regeneration is primarily of two types. The most widely recognized type of regeneration is compensatory hypertrophy via tissue proliferation. In this form of regeneration mature, highly differentiated cells are stimulated to

become mitotically active following an injury and replace all or most of the tissue lost. The most common example is liver regeneration. If as much as two thirds of the liver is lost due to accidental injury or surgical intervention, the remaining hepatocytes and vascular endothelium will proliferate until most of the tissue lost is replaced by new tissue which is both morphologically and physiologically similar to undamaged tissue. Little or no differentiation occurs and regeneration is accomplished entirely by proliferation of cells already differentiated to form liver. The epidermis of the skin often shows tissue regeneration by increasing its mitotic rate and rapidly correcting or covering a cutaneous defect, therefore reestablishing the first line of defense for the underlying damaged tissues. Thus the liver and the mammalian skin are analogous in their repair processes, doing so by the multiplication of pre-existing differentiated cells.

The second major type of regeneration is epimorphic regeneration, or the establishment within wounded tissues of a mass of undifferentiated cells (blastema cells) which proliferate and differentiate (or redifferentiate) to replace the lost tissues. This type of regeneration mirrors the embryonic process of tissue formation whereby all the complex, highly differentiated tissues of a region are formed from similar embryonic mesenchymal cells. The most visible evidence of this form of regeneration is the limb and tail regeneration seen in amphibians and the taste barbules of catfish (Goss, 1956a). Many development biologists believe that it is through extensive dedifferentiation of existing differentiated cells that the blastema arises by a process called metaplasia (Slack, 1980). It is then that this mass of cells undergoes redifferentiation to form the regenerate which is superficially analogous to normal limb development in ontogeny.

Epimorphic regeneration occurs in three distinct phases. The first phase is wound healing, which involves two simultaneous events. The epithelium on the injured surface rapidly proliferates and migrates over the denuded region. Underneath the epithelium, cellular exudation takes place which destroys any injurious agents present and dissolves and removes all necrotic cells, debris and fibrils. This phase may last several days during which time there is an increase in hydrolytic enzymes, such as cathepsins, acid phosphatase, peptidase, dehydrogenases, and collagenase (Schmidt, 1968). At the same time cells capable of proliferation replace the lost or damaged cells and thereby bind together the tissue walls separated by the wound. In most excised wounds only three types of cells are active; fibroblasts, vascular endothelium, and epithelium. The fibroblasts and the vascular endothelium produce the connective tissue and blood vessels to bind wound edges together and the epithelium proliferates and covers the wound. In most wounds the young richly vascularized fibrous connective tissue produced contracts and persists indefinitely as scar tissue (Peacock and Van Winkle, 1976). This last event however does not occur with regeneration. The capacity to regenerate substantial parts of the body requires more than the production of new molecules and cells. Cells must be assembled into tissues, and tissues into organs, with the restoration of form and function.

The second phase of epimorphic regeneration is termed dissociation and is the stage which distinguishes regeneration from wound healing. The cartilage, connective tissue and muscle break up or dissociate (dedifferentiate) into mononucleate cells which are indistinguishable from each other and as such form the mesenchymatous cells of the blastema (Slack, 1980).

Various theories exist on the origin of the blastema cells including cells derived from the damaged tissues, leucocytes, or differential stem cells present in all tissues. Most authors maintain that the source of blastema cells are the tissues bounding the wound. Butler's (1933) irradiation experiments both with partial radiation in situ and with transplantation of unirradiated limbs to radiated hosts showed clearly that the cells used in the regeneration processes must arise locally and do not originate in other parts of the body or in the blood. Through elaborate mitotic studies (Chalkley, 1956), electron microscopic (EM) studies of regenerating muscle (Hay, 1959), and auto-radiographic studies (Hay and Fischman, 1961) it was demonstrated that blastema cells arise locally and from stump tissues. Hay (1959) through extensive EM studies without isotopic labelling of cells showed that the muscle in the stump does dedifferentiate and form blastema cells. Also Hay and Fischman (1961) used autoradiography to detect incorporation of tritiated thymidine by the cells of the regenerating newt limb during blastema formation. They saw DNA synthesis 4-5 days after amputation in the dedifferentiating muscle, endomysium, epimysium, periosteum, nerve sheaths and loose connective tissue of the stump. The blastema cells appearing subsequently were labeled; therefore, it was deduced that they derived from internal tissues. Animals given label prior to amputation showed labeled blood cells but none of the label appeared in the blastema suggesting that blood cells are not the source of blastema cells. However Becker and colleagues (Becker and Murray, 1967; Becker, 1972; Becker et al. 1977) stimulated frog red blood cells with very low electrical currents and found the erythrocytes would revert to blastema cells, evidence which supported their earlier histological observations of blastema cells forming from a blood clot in bone (cited in Schiefelbein, 1978).

The last phase of regeneration is progression or differentiation. As a result of distal migration and proliferation, the population of mesenchymal-like cells or blastema accumulate beneath the thickened wound epithelium. Here the blastema cells increase in number and following similar embryonic patterns, differentiate into the missing tissues of the organ.

Studies using axolotl tissues labelled by tritiated thymidine and triploidy (Steen, 1968; Namenwirth, 1974; and Dunis and Namenwirth, 1977) have shown that extensive metaplasia occurs among the different internal tissues, all of which are derived from the embryonic mesoderm. These studies also indicated that some cells are more stable to cell type than others. Limb chondrocytes are intrinsically stable with respect to type for they give rise to blastema cells which then exclusively differentiate into chondrocytes (Namenwirth, 1974). However certain cells from transplanted limb muscle can become chondrocytes. Grafts of a particular marked tissue into an X-irradiated limb followed by amputation of the limb through the graft region leads to the formation of a regenerate entirely composed of graft-derived cells as seen by the retention of graft marker in the various cell types (Maden, 1979b). Myotubes are seen to arise from dermis, muscle and nerve sheath (there is conflicting evidence on the origin of myotubes from cartilage). Chondrocytes arise from dermis, muscle, cartilage, and nerve sheath, epidermis arises only from epidermis. To date there exists no biochemical, isotopic, or morphological data which implicates epidermal cells as possible blastema precursors or that epidermal cells can form other cell types (Slack, 1980). Even though some cell types are stable, Weiss (1925) and Thornton (1938) showed that limbs from which bones were removed prior to amputation, regenerated limbs with bones in the

correct position. It may be postulated then that these bone cells arise from muscle cells or cells associated with muscle.

From this review of regeneration it is evident that during the first phase the epidermis is the most mitotically active tissue. Emerging from an initial state of relative quiescence, the internal tissues become the most active during the dedifferentiative phase. The tissues of the blastema appear equal in mitotic activity to the dedifferentiating tissues, but the cells of the newly forming tissues become more mitotically active than the blastema cells (Bodemer and Everett, 1959).

The formation of the blastema is dependent upon a number of factors. One is the development of a wound epidermis. Rose (1944) was able to induce regeneration in the forelimb of adult frogs by maintaining dermis-free contact between the migrating mature-type epidermis and internal stump tissues. Apparently the wound epidermis-blastema interaction is required for distal outgrowth of the blastema as well as differentiation of the distal structures (Faber, 1965; Stocum, 1968; Stocum and Dearlove, 1972).

Systemic factors as well as local are required for successful regeneration. The dependence of limb regeneration on nerves has been documented in a series of reviews (Singer, 1952; 1974; 1978). The same initiation of regeneration seen in the adult anuran with the wound epidermal-mesodermal interaction is also seen when there is an increase in the neural supply. Regeneration will occur in the normally nonregenerating limbs of frogs with an increased quantity of nerves (Singer, 1954). Limb regeneration in the newt occurs when the amount of nervous tissue per wound size (nerve/amputation ratio) is above a certain threshold value but fails when the ratio is subthreshold.

Dresden (1969) found that denervation markedly decreases the DNA, RNA, and protein in the blastema in vivo and blastema cultured in vitro. Following denervation of the blastema, DNA synthesis declines (Singer and Caston, 1972) and the mitotic index falls to zero (Singer and Craven, 1948); newt brain extract injected into the blastema reinitiates DNA synthesis (Jabaily and Singer, 1977) though the extracts lose their activity if heated or digested with proteolytic enzymes; and after X-irradiation a mitotic block is incurred (Maden and Wallace, 1976; Maden, 1979a). A neurotropic factor is needed for the cell proliferation of blastema formation but not for the differentiation of blastema cell into muscle, cartilage, and other tissues of the new limb (Powell, 1969).

The elucidation of the neurotropic factor is being heavily pursued at this time and is particularly interesting since this factor is shared by embryonic tissues (Singer, 1965). A prime candidate is neurotropic growth factor (NGF) isolated from brain tissue (reviewed by Gospodarowicz and Mescher, 1980). It is a protein which arises from the basic protein of myelin and possesses mitogenic activity for a wide variety of cells derived from mesoderm. An injection of NGF in denervated newt limb stumps can induce blastema cells to proliferate with a mitotic index approaching that of innervated limb stumps. Likewise injections of NGF into the amputated limbs of adult frogs leads to hypomorphic regenerates with well-differentiated cartilage and proximal skeletal muscle very much like the regenerates produced by Singer's (1954) augmentation of nervous supply to adult anurans. NGF also stimulates myoblasts to divide and vascular endothelium to develop in vivo and in vitro. The metabolic changes induced by NGF are all required for regeneration thus NGF possesses characteristics of the classical neural agent promoting limb

regeneration but whether it is the agent has yet to be shown (Gospodarowicz and Mescher, 1980).

After Singer (1952) demonstrated the need for a threshold mass of nerve tissue for regeneration, the evidence over the past 20 years indicates the factor supplied by the neural tissue was a negative electrical environment (Singer, 1978). The identification of the current as a neural factor of regeneration was made recently by Rose (1978) who obtained regeneration in denervated salamander extremities by applying the proper current levels and polarity. The interactions between this current and the neurotropic growth factor have not been demonstrated. Regenerating tissues demonstrate a high electronegativity and experimentally such an electrical movement has been shown to stimulate regenerative growth in species not normally possessing such a capability (Becker, 1972; Becker and Spadaro, 1972; Smith, 1974). Becker and Spadaro (1978) suggest that the electrical stimulation is more one of power density than polarity. Both positive and negative currents have been seen to induce a three-fold increase in the cell population of cultures of human fibrosarcoma (Becker and Esper, 1981). Simon et al. (1980) have also reported an increase in number of metastases with experimental tumors in rats by cerebral electrostimulation.

A second class of systemic factors involved in regeneration are the hormones. The classical work was done by Schotte in 1926 who hypophysectomized newts and observed that they could not regenerate and concluded that pituitary hormones are required for regeneration. Schotte and Wilbur (1958) suggested that the trauma of amputation induces production of adrenocorticotrophic hormone (ACTH) which stimulates the synthesis of adrenal steroids thought necessary for regeneration and Bromley (1971) showed the preferential accumulation of adrenocorticosteroids near an amputation surface.

However prolactin and somatotrophic hormone (STH) have been shown to be more effective than ACTH in promoting newt limb regeneration (Tassava, 1969); and Kent (1977) suggests that the hormone most critical to regeneration is prolactin. Additionally, McCartney and Mulcare (1973) found that prostaglandins would stimulate a regeneration response in the anura.

Apparently some combination of prolactin, growth hormone, thyroxine, adrenocorticosteroids, and insulin is required for optimal tail and limb regeneration in the newt. Their function is tied to the hormone somatostatin, a hypothalamic release inhibiting hormone, which inhibits the secretion of GH, TSH, and prolactin and reduces blood glucagon and insulin (Yen et al., 1974). Limb or tail regeneration was retarded in adult newts given somatostatin prior to amputation and two of the newts failed to regenerate at all (Vetamany-Globus et al., 1977). Since the majority of these hormones are general stimulators of cell growth either by directly increasing the uptake of metabolic precursors or by mobilizing the body's stores of needed building materials, the lack of regeneration after inhibition of their secretion may simply be due to inadequate growth requirements to the mitotically active tissues. It can be suggested that most of these hormones serve a more supportive than a regulatory role in regeneration.

Mammals, including the human, can replace substantial lost portions of the liver, spleen, and pancreas while retaining most of the structural and functional integrity of the organs (Varricchio et al., 1977; Rehns, 1979). Compensation for what is missing occurs by an enlargement of the portion of the organ that is left (compensatory hypertrophy). Other examples of mammalian regeneration are scattered. Mammalian muscle will regenerate from a minced muscle mass to a functional state with tendon attachments but its

morphological integrity is not reconstituted (Carlson, 1970). Entire muscles, morphologically and functionally perfect, are formed in a regenerating newt limb by epimorphic regeneration involving a blastema (Carlson, 1979). In the mammal, tissue regeneration of muscles is rapid, occurring without a blastema. The tissue end product is smaller and less well organized than a normal muscle and its morphogenesis seems to require the function of the limb. Muscle regeneration in the mouse, rat, and guinea pig is seen in both transplanted whole muscles and minced muscles (Carlson, 1979).

The mechanism of restoration of the muscle structure is controversial. (Sloper and Partridge, 1980). Current theories are that the new muscle cells arise from satellite cells (Zacks and Sheff, 1977; Snow, 1977) or from cells derived by dedifferentiating multinucleated muscle cells into single myoblasts (Schmalbruch, 1977; Hay, 1959), or a third hypothesis is that myoblasts arise from satellite cells derived from the damaged multinucleated cells (Reznik, 1969a, 1969b). The experimental analysis of myogenesis is likely to be even more difficult now since it has been shown that skeletal muscle cells can arise, in vitro, from such diverse tissue types as fibroblasts, neurons, and glia (Lennon and Peterson, 1979).

A similar pattern exists for cartilage regeneration. In the tailed Amphibia cartilage regeneration occurs epimorphically (Kent, 1977). Cartilage will also regenerate by direct differentiation from the undifferentiated layer of periosteum (or perichondrium) without the formation of a blastema at the cut end of bones in frog limbs (Goode, 1967; Polezhaev, 1968), in amputated mouse digits (Schotte and Smith, 1959; Scharf, 1961) and in defects made in rabbit ear cartilage (Wasteson and Ohlsen, 1977; Skoog et al., 1972). Also, cut bones of both adult and immature rats have been stimulated to regenerate

in part by exogenous agents such as CaCl_2 and trypsin treatments, vitreous nervous tissue preps, and cortisone hormone treatments (Scharf, 1961, 1963; Polezhaev, 1972).

The healing of fractures in mammals is histologically typical of a regenerative response including the formation of a blastema derived from mitotic activity of the periosteal and endosteal stem cells despite the lack of good innervation. However when bone is stressed it produces electrical potential and the supply of a negative current to the endosteum of bone causes it to proliferate. Therefore bone regeneration (osteogenesis) may be due to the production of these currents by the bone and this information applied clinically has augmented healing of bone fractures (Becker et al., 1977; Becker, 1979). It is also interesting to note that prolactin appears to enhance the morphological response to the current (Becker, 1972).

A distinctive type of mammalian regeneration is seen in the cyclic replacement of antlers. Antlers are regenerated each year by the activation of an amputation plane which pushes off the old antler and regenerates the new (Goss, 1961). This type of regeneration is called physiological or repetitive; another example is the endometrial cycle. However antler regeneration is distinguished from basic repetitive regeneration in that there exists at the tip of the growing antler an apical cap similar to that of the apical ridge of the developing chick limb or blastema cone in Amphibia. Goss suggests that morphogenetic fields are present in the antler very similar to the limb model of French et al. (1976). However, it is interesting to note that this regeneration is not nerve dependent.

Mammalian limb regeneration is nerve dependent. However in neither embryonic nor postnatal animals do amputated limbs normally regenerate.

Mizell and Issacs (Mizell, 1968; Mizell and Isaacs, 1970) initiated regeneration in the opossum pouch young by increasing the nervous supply to the limb with tadpole forebrain and sibling opossum cerebral cortex. The role of nerves is also illustrated by the work of Becker (1972) with inducement of partial limb regeneration in rats by applying appropriate levels of electrical stimulation. Neufeld (1980) has shown partial blastema formation after limb amputation in adult mice by repeated skin removal and exposure to a saturated solution of NaCl. Deuchar (1976) has demonstrated regeneration of amputated limb buds in 11.5 day old rat embryos grown in culture. Becker (1972) passed an electrical current through the foreleg of a rat after amputation and gained partial regeneration from shoulder to humeral elbow. Extending these studies to the human, currents passed through the ends of fractured bones, for as long as 16 years, stimulated the ends to grow together forming strong useful bones thereby knitting fractures that had previously failed to heal even after extensive surgical procedures (Becker et al., 1977). Hyaline cartilage at the epiphyseal ends of long bones is capable of proliferation and healing partially the defects made in it, a response enhanced by changes in the electrochemical environment (Baker et al., 1974). With Becker's studies on rat bone repair after denervation of the limb he found that the repair was due more to the regeneration of the Schwann cells than the nerve. Becker (1979) suggested that a major factor in mammalian or amphibian limb regeneration and osteogenic bone repair is the electrical current produced as a result of the injury or exogenously supplied.

The phenomenon of finger regeneration in children has been observed (Illingworth, 1974). The data exist primarily for children to 11 years of age but two examples of young adults are known. If the distal phalangeal segment is amputated distal to the joint and no surgical intervention occurs, a complete

segment will regenerate with fingerprint and fingernail. Whether this occurs via tissue regeneration or epimorphic regeneration is not known. It is obvious that the replacement of the fingernail and fingerprint would have to be more complex than simple tissue proliferation. One can postulate that the lack of regeneration after surgical intervention may be due to the prevention of interaction between a wound epidermis and the underlying mesoderm as mentioned previously (Stocum, 1968; Stocum and Dearlove, 1972). With surgical repair the mature epidermis proximal to the wound is pulled over the damaged tissue and seals the wound so that wound epidermal proliferation cannot occur. In the newt and other regenerating animals a modified epidermis persists for several weeks as a wound epithelium which is a thickened epithelium lacking a basement membrane. Regeneration will not proceed if this wound epithelium is absent (Thornton and Steen, 1962). Neufeld (1975) demonstrated that in mice wound contraction minimizes the area to be covered by wound epithelium so that the epithelium converted to normal epidermis within a week. Therefore with salt treatments and surgically removing the skin a partial blastema did develop but the lost distal structures did not develop. Person (1979) showed partial regeneration in the adult rat forelimb by the use of a muscle flap to cover the wound but no blastema was formed.

A natural variation exists in the manifestation of regenerative capacity (Liozner, 1974). For example, the cranial bones will regenerate in rabbits but not in rats or dogs. These bones will regenerate in all animals when young, but they will not regenerate in adult animals of the species mentioned, a characteristic of most regenerative phenomena in mammals. Children and other young mammals can replace lost cranial bones from bone fragments but adults cannot (Liozner, 1974). In rabbits the ventral skin of the ear can form

specific epithelial derivatives (hair and glands) during the healing of wounds, whereas on the dorsal surface of the ear they are replaced sporadically (Liozner, 1974). This variation in regenerative capacity with age, species, and region of the body may indicate that even though the tailed amphibia are the most successful, no species has completely lost the capacity to regenerate in all its body parts.

True epimorphic regeneration, as typified by the newt limb, is always preceded by the formation of a distal mass of mitotically active, embryonic-like blastema cells. Although blastema formation in the neonatal opossum limb (Mizell and Isaacs, 1970) and partial formation in adult mice by surgical and salt treatments (Neufield, 1980) has been observed, the only histologically verifiable example where the blastema serves to complete restoration of lost tissues is that of the rabbit ear tissue regeneration system.

Rabbit Ear Tissue Regeneration

The phenomenon of rabbit ear tissue regeneration was presented in a dissertation by the Russian scientist, Markelova in 1953 (translated to the English by Vorontsova and Liosner in 1960) with extensive histological evidence. Rabbits can replace tissues lost in an ear punch with the same tissue types and in the same relationship. This replacement has been termed a true epimorphic regeneration since the regenerated tissues appear to arise from a mass of undifferentiated cells which accumulate underneath the wound epidermis by day 14, post punching.

After a full-thickness wound has been made in the ear of an anesthetized rabbit the repair of the area is begun by simple wound healing. A scab forms over the edges of the wounded tissue, the epidermis thickens and then migrates under the scab to completely cover the damaged area. Beneath this wound

epidermis, which is several layers thick, the damaged tissues at the edge of the wound are removed and in their place accumulates fibroblasts and a new mass of cells very similar to undifferentiated mesenchymal cells. This new mass of cells, the blastema, or regeneration bud, (which can be seen as a macroscopically edemic-like swelling by day 14) is thought to arise by dedifferentiation of pre-existing cells. Blastema cells proliferate apically and redifferentiate basally to eventually fill in the missing part. The regenerated tissue consists of both dorsal and ventral skin, elastic cartilage, fibrous connective tissue, vascular tissue, nerves and eventually hair.

The primary aims of this study are to; (1) determine the optimum conditions for rabbit ear tissue regeneration and to understand some of the basic tissue interactions required for this process; (2) survey other mammals for the capacity of ear tissue regeneration; and (3) make a comparison between the ear tissue regeneration system and embryonic systems of development and wound healing by looking at specific biochemical changes during ear tissue regeneration which might also occur in these other two systems.

The rabbit ear tissue regeneration system is the best available model for studying mammalian regeneration. It is extremely difficult to make comparisons in results between such diverse species as the newt and the mammal. The rabbit provides a mammalian model for regeneration; in fact, the regeneration of the rabbit ear tissues is the only known example of epimorphic regeneration in mammals outside the regeneration of the terminal digit segments of children. Advantages to using this system include: The simplicity of the process due to the accessibility of the ear, the requirement of little equipment for tissue biopsy, the subjection of animals to a minimum of stress during punching, the lack of extensive inflammation with wounding, (so the risk of

infection is near zero, i.e. no cases have been reported), the availability of double sampling per animal since each ear responds independently of the other, and it can be performed on various other animals without disabling them or removing them from other non-interfering research programs.

For our comparisons between the regeneration process and embryogenesis, the rabbit is an excellent model since it is one of the most widely used animals for studies in mammalian reproductive physiology and embryogenesis. The focus of the research performed in our laboratory has been on the reproductive physiology and early embryogenesis of the rabbit so we are familiar with the normal characteristics and changes seen in these systems.

Since the ear is composed of a central layer of cartilage the opportunity is provided to use cartilage matrix changes as monitors of differentiation. Various studies have been performed on the changes in matrix molecules during differentiation, and the use of these molecules as indicators for differentiation (Robinson and Dorfman, 1969; Linsenmayer et al., 1973b, c; Mayne et al., 1976; Hasty et al., 1981). Thus, comparisons can easily be made between the changes seen with regeneration and those seen in embryogenesis.

These studies of the rabbit ear tissue regeneration system were designed to examine specifically a number of basic parameters including the pattern of regeneration both chronologically and morphologically, the effect of age, hormonal status, location of the punch on the ear, and biochemical changes within the matrix of the connective tissues (which might offer clues to substantiate the process as a true epimorphic regeneration event). We examined how this system of rapidly proliferating cells might be similar to early embryogenesis and we also looked at the capacity to regenerate ear tissues among other mammals.

A SURVEY OF RABBIT EAR TISSUE REGENERATION

Introduction

The replacement of the original tissue types into holes cut in the ears of rabbits is one of the best examples of true epimorphic regeneration in mammals. Rabbit ear tissue regeneration (RETR) proceeds experimentally after a one-centimeter diameter hole is punched in the cartilaginous region of the ear (Carlson, 1978b).

The chronological events and blastema morphology of the system have been established (Grimes and Goss, 1970; Goss and Grimes, 1972, 1975) and the histological progression described in detail (Joseph and Dyson, 1966a). There is evidence that the steroid sex hormones exert an effect on the rate and complexity of the regenerative process. Tissue replacement occurs faster in males than in females, orchidectomy decreases the regeneration rate, and treatment of females with testosterone pellets implanted in the ear or systemic injections of synthetic androgens stimulates regenerative growth (Joseph and Dyson, 1965; Dyson and Joseph, 1968). Progesterone supplementation also stimulates regeneration in both sexes as does low levels of estradiol in females. High estradiol levels are inhibitory in both sexes (Dyson and Joseph, 1971).

Size and shape of the wound seem to make little difference to the success of regeneration. Whereas the experimental regeneration procedure typically involves round holes of five to ten mm in diameter, Joseph and Dyson (1966a) also obtained good regeneration for square holes of up to three sq cm in area and Grimes (1978b) showed that wounds in the form of a Greek cross, hour glass, half circle, crescent, and triangle all regenerated. Completely

amputated ears will not regenerate, although Grimes (1978b) demonstrated significantly better growth when the transection was proximal on the ear as compared to distal.

Goss and Grimes (1972) found that the graft substitution of belly skin for ear skin would not support cartilage regeneration when the ear was subsequently punched through the transplanted area. They also showed that if the sheet of cartilage was removed from the ear and the resultant area punched through the remaining two layers of normal ear skin, regeneration never occurred. They concluded that the proximity of a healing wound in the overlying ear skin is required for cartilage regeneration, and that skin from elsewhere on the body cannot support ear cartilage regeneration (Goss and Grimes, 1972). Grimes (1976) removed both skin (from one ear surface only) and cartilage and saw only sporadic if any cartilage regeneration. Robertson (1981) saw an alteration in the morphology of the regenerated cartilage layer in rabbits given 12% ethanol ad lib. In these alcohol treated animals regeneration ensued with a severe edema which did not alter the overall rate of closure but histologically may have altered the normal paths of the blastema cells. The newly regenerated cartilage was not replaced in continuity with the old cartilage layers.

Grimes (1973, 1974b) showed that X-irradiated cartilage would support ingrowth of integument, but not cartilage, from the margin of ear holes. In a series of abstracts, Grimes and colleagues (Grimes and Goss, 1972; Grimes, 1973, 1974a, 1974c, 1976, 1977, 1978a; Grimes and Rowland, 1978; Grimes and Kulis, 1979) examined several aspects of the interaction of skin, nerves, and cartilage in rabbit ear regeneration. They reported: (1) Integument ingrowth into an ear hole is not dependent on the presence of living cartilage, as

demonstrated by skin regenerating into holes punched in areas where normal ear cartilage had been replaced with frozen-killed cartilage. (2) Animals with irradiated integument regenerate well while those with irradiated cartilage are not covered by integument nor do they regenerate or undergo chondrogenesis. (3) Partial (and slow) regeneration can occur when the hole's margin contains non-ear cartilage, such as from the sternum's xiphoid process. (4) The ear regenerative process is curtailed by covering a hole's margin with mature integument by apparently blocking the epidermal-chondrogenic interaction. (5) Denervation of the ear has no blocking or retarding effect on regeneration. (6) Ear skin, transplanted to other sites (e.g. tail) does not support regeneration when these structures are transected and, (7) the addition of another sheet of cartilage to provide two layers in the wound area results in only one regenerated cartilage layer.

Goss and Grimes (1975) noted from histological studies that downgrowths of long tongues of epidermal cells into the underlying dermal connective tissue distinguished regenerating ears from nonregenerating and that dogs and sheep with similar ear punches do not form these epidermal tongues and do not regenerate. They did not find epidermal tongues in rabbit ears prevented from regenerating by prior removal of the cartilage. From these observations it can be suggested that since the cartilage is indispensable for ear regeneration, an epidermal- chondrogenic interaction may be necessary to prevent the normal tendency of the skin to form a scar. With ear tissue regeneration an organized cartilage matrix forms within the blastema contiguous with the old cartilage. The morphological differentiation of this recognizable cartilage matrix from blastema tissues provides a valuable system for studying adult cartilage regeneration and repair.

Various biochemical changes occurring within the blastema of the rabbit ear as it differentiates into adult tissues have been measured recently. Grimes (1981) showed histochemically the presence of hyaluronate in the path traveled by the blastema cells and with colorimetric studies demonstrated a marked increase in hyaluronidase activity coinciding with both the cessation of this migration and the onset of chondro- and fibrogenesis. Hasty et al., (1981) looked at changes in glycosaminoglycans (GAG) synthesis at various stages of regeneration. They found a change in the position of sulfation of chondroitin sulfate which mimics those seen embryonically, i.e. a shift in the ratio of chondroitin-4 and chondroitin-6 sulfate. They also found an increase in hyaluronic acid at the time of cartilage differentiation which is in contrast to that seen in the newt. Further studies on matrix components in the ear are found in Chapter IV.

Smith and Kang (1981) looked at in vitro collagen synthesis by regenerating ear tissues in culture and showed the shift in types of collagen found in the cartilage from Type II in normal cartilage to Type I. The unusual Type I-Trimer was seen at the blastema stages and a conversion back to Type II was observed with increased cartilage differentiation. These results have been confirmed and extended in vivo (Chapter IV). It is known that chondrocytes in culture often lose their characteristic morphology and switch their collagen type production to Type I-Trimer. From the discovery of this type collagen in blastema tissue one can hypothesize that the mature ear chondrocytes must have dedifferentiated since the normal cell type fibroblast found in connective tissue never produces Type I-Trimer.

These studies were undertaken to provide a framework for continued research on the rabbit ear tissue regeneration system. To monitor either biochemical, physiological or morphological phenomena certain critical

parameters regulating the process and a time scale for closure and regeneration had to be determined. This information would allow the system to be used optimally, i.e. to use those parameters which yielded the greatest regenerative success.

A number of elements of the regeneration system such as the time required to close and regenerate; the histological progression; physiological factors which regulate the success of regeneration such as age, sex, hormonal status; the presence of other wounds on the same or contralateral ears; the position of the lesion on the ear; and the ability of other complex tissues to participate in the regeneration event were monitored. Valuable information concerning the local as well as systemic factors required for regeneration was gained. Some of these findings have been published (Williams-Boyce and Daniel, 1980).

Materials and Methods

The experimental animals were New Zealand White rabbits, unless otherwise indicated, of both sexes, ranging in age from two weeks to three years. For analgesia and tranquilization, the animals were given an IM injection of Innovar-Vet (Pitman-Moore, Inc.) at a dosage of 0.3cc/kg body weight. The ears were shaved and holes punched in them with a steel gasket punch or cork borer ranging in diameter from five to ten mm, with size dependent on the age of the rabbit. Punches were made in areas of both ears between the medial ear artery and the marginal ear veins, where there are few major vessels (Figure 1). Animals were also punched on the outer margins of their ears which yielded a fractional hole with an open side. Any excess bleeding was stopped with slight pressure from a gauze pad. Tissue measurements or collections were made at varying intervals throughout the process of wound

closure and regeneration and the results are reported per ear since each ear acts independently of the other. These basic methods were followed throughout the subsequent studies.

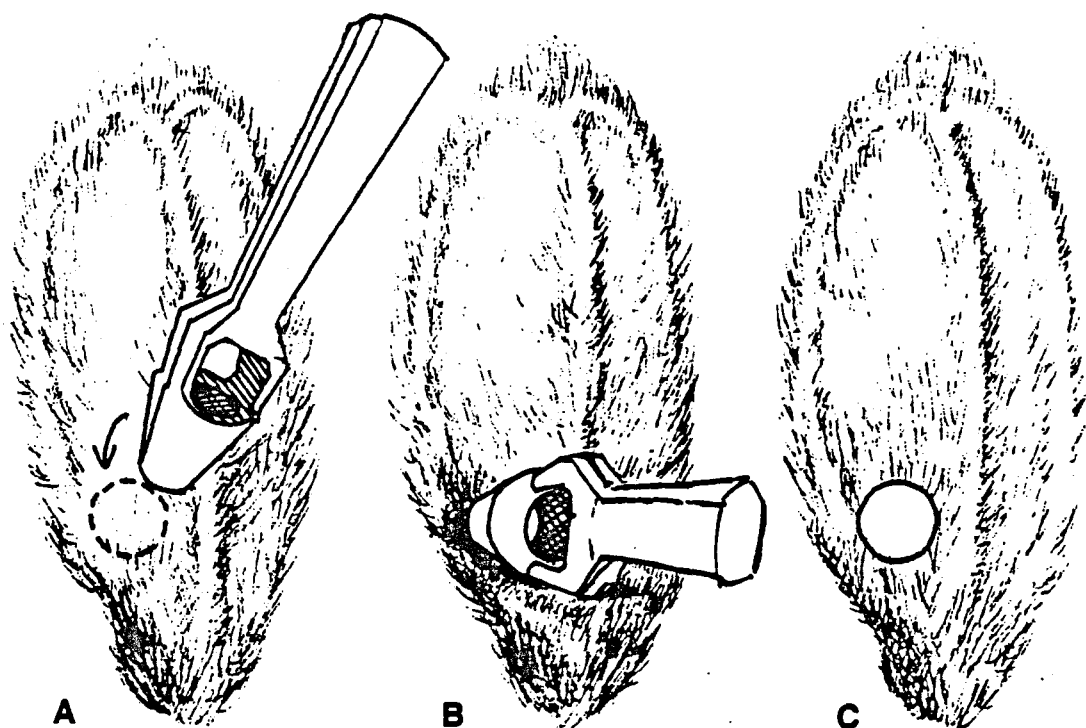


Figure 1. Procedure for punching rabbit ears. An area is selected in the proximomedial region of the ear between the large medial artery and the marginal ear veins. The biopsy is made with a hand held steel gasket punch, yielding a hole 1 cm in diameter.

For the kinetics of closure, the size of the hole was recorded three times weekly, measuring the diameter of the hole along both the long and short axis of the ear, and averaging the two measurements since the tissue was frequently replaced eccentrically. Animals that were subjected to surgery were either (1) tranquilized with Innovar-Vet (0.3 cc/kg body weight) and ten minutes later anesthetized with Nembutal (sodium pentobarbital, Abbot Labs) 1.5 mg/kg or to effect but not to exceed 15 mg or (2) anesthetized with xylazine (Haver-Lockhard) at a dosage of 10 mg/kg.

Hormonal treatment. Eight mature does were bilaterally ovariectomized via a midline abdominal incision using standard support techniques. After surgery, the animals were left untreated for two weeks to allow for metabolism of endogenous ovarian steroids. They then received ear punches and were injected daily with testosterone, 3 mg/kg of body weight in corn oil (subcutaneously) or with corn oil alone as control. The data for all test groups were plotted as percentage of the original punch remaining at weekly intervals.

Skin transplantation. Six Dutch Belted rabbits (12 months old) were employed for this experiment since they have both black and white pigmented areas of skin. After anesthetization their ears were shaven and circular pieces of dorsal, black-pigmented short-haired ear skin one cm in diameter were removed from the underlying cartilage and connective tissue. An equal-sized (one cm) piece of white, long-haired skin from the suprascapular area of the back was removed and sutured to the wound in the ear with a Ford interlocking pattern (Walker et al., 1980). The grafts produced in this method were covered with Vaseline to keep them moist. The back to ear grafts were placed in either of two positions, with the back hair in the same position as the ear hair, or the back hair rotated 90 degrees to the ear hair. One month post-surgery healing appeared complete on the ears. The animals were divided into two groups, one group remained as untreated controls and the ears of the second group were punched with a five mm diameter cork borer through the transplant region. The animals were observed for up to four months thereafter.

Pigmentation replacement. Three Dutch Beted rabbits with two-color hair patterns on their ears were punched at various color sites; all white, all

black, or at a boundary where white and black met. The regeneration was allowed to progress until hair formation was complete at four to six months.

Toe transplantation. To examine the question of whether complex tissues can regenerate if placed into the environment of the ear, the fifth carpal digits of six young male rabbits were transplanted to the ears of the same animals. An elongated hole of about five x ten mm was cut in one ear of an anesthetized shaven animal. The small toe from the same side was then amputated above the second joint, the nail removed and the skin cut around the periphery of the toe so that both dorsal and ventral skin layers could be flattened. The toe was positioned in the ear hole and held in place by suturing the edges of the dorsal toe skin and ear skin together and then repeating the process on the ventral ear surface. The result was a preparation where skin covered both surfaces and internal toe tissues (bone, muscle, connective, etc.) were thereby suspended in a space in the internal ear tissue and in contact on all sides with it.

Alternatively, internal toe tissues were exposed by skinning the amputated toe and then inserting the internal tissues into a pouch made in the ear. The pouch was made by first cutting a short incision in the dorsal skin and, with blunt forceps, forcing it away from the underlying cartilage. The ear incision was closed with small sutures after the toe tissues were in place. The toe amputation site was closed by suturing the adjacent skin surfaces. After two weeks, allowed for healing, holes were punched in these ears so as to cut across part of the transplanted toe region. X-ray photographs were taken of the ears after the toe transplants were completed, again after the holes were punched and again 60 days later to appraise whether the bone had regenerated or not. The ear contralateral to the one receiving the toe

tissue transplant was also punched in each case to provide a control as evidence of a particular animal's capacity for regeneration.

Results

A total of 26 animals (52 ears) including adult males, females, and juveniles were used to confirm earlier reports in (Grimes and Goss, 1970; Goss and Grimes, 1972, 1975) determining the length of time needed for regeneration of the tissues lost in an ear punch, and the frequency of regeneration (Figure 2). The broken line represents the entire study group, while the solid line represents those 19 ears which successfully completed closure within the ten week period of the experiment. Of those 19, 18 were closed by day 49. It is obvious that inclusion in the data of the holes which failed to regenerate causes the closure curve to be skewed. For this reason, in the remainder of the studies, the data will only show regeneration in those ears successful in closing their wounds, but the ratio of closures to non-closures will be included.

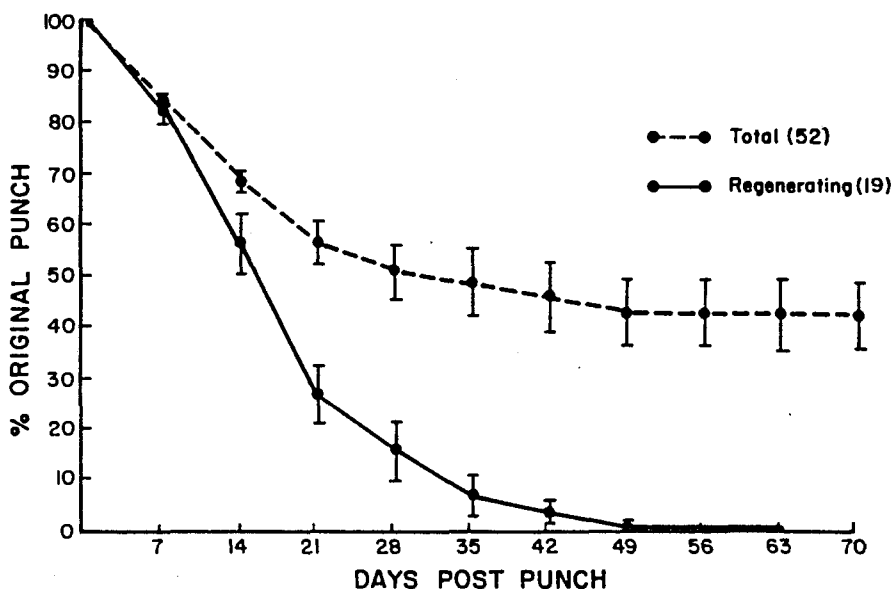


Figure 2. Closure of holes during rabbit ear tissue regeneration. Solid line represents those ears which completed closure within 10 weeks, broken line includes all samples. Vertical lines represent standard errors of the mean. Values in parenthesis are numbers of ears.

Adults: hormonal stimulation. Joseph and Dyson (1965, 1966b) and Dyson and Joseph (1968) reported that the growth of the regenerated tissue was significantly faster in males than in females. To seek confirmation of this observation the data from our initial study (Figure 2) relevant to the sex of the adult animals was reanalyzed (Figure 3). Of the eight ears of male animals in the study, the holes in five were closed by day 42 and the last one by day 56. Of the females, three of 14 were successful in completing closure, two of these as early as day 35. Thus in these studies there was no difference in closure time between males and females. However, males had a higher frequency of regenerative success than did the females; 20% of the ears of female rabbits regenerated within ten weeks, 75% of the ears of males did so.

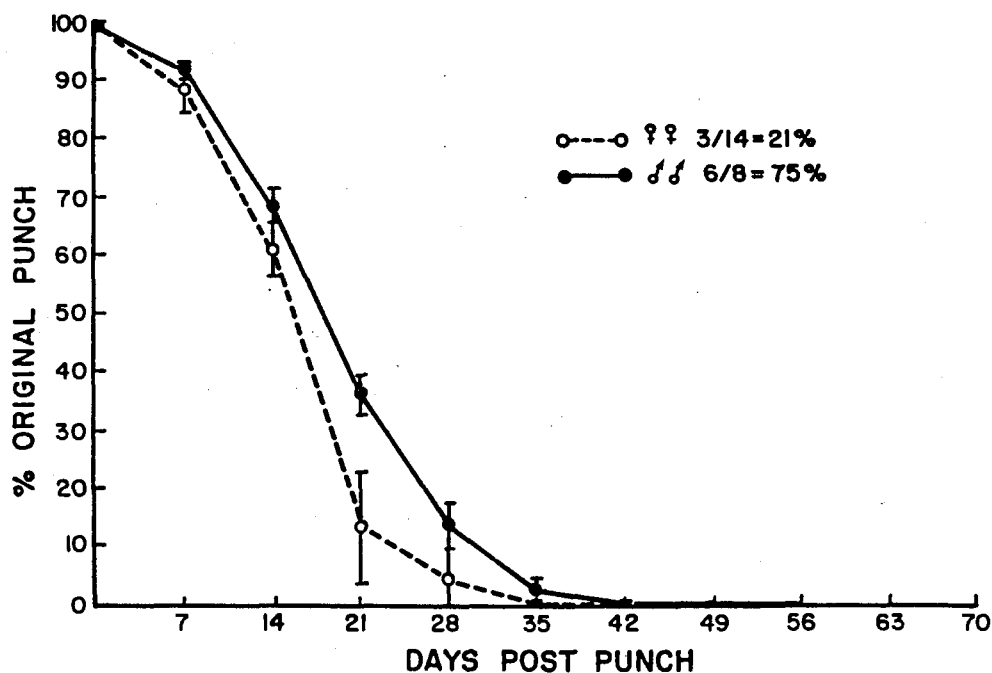


Figure 3. Comparison of ear hole closure between males and females. Ratios are number of ears closed out of number of ears in test group.

The females used in this study were in variable reproductive states, i.e. pregnant, pseudopregnant, estrus, or anestrus. It was noted, however, that among the females only those in estrus or anestrus were successful in completing regeneration of the lost tissues. Because the sample here was so small we punched the ears on another group of pregnant and lactating does. Of eight holes punched in ears of pregnant rabbits one day after breeding, three closed within the 32 day gestation period and three more by day 40. For lactating animals, of six holes punched one week after partuition, only two closed within the 60 day observation period.

To determine if the difference seen between males and females was due to the presence of androgenic hormones, testosterone was administered to castrated females. Figure 4 shows the results of this experiment. There was little difference in the time required for the replacement of lost tissue in both the controls and experimentals. The control, corn oil-injected, animals that regenerated completed the replacement by day 42. Six of the seven holes in ears of the experimental, testosterone-injected, animals completed their replacement by day 42, the seventh completed by day 56. The major difference between the two groups lies in the percentage of animals which were successful in regeneration. Of the castrates which were testosterone injected, 88% of the ear holes regenerated while only 50% of the ears of castrated, corn oil-injected animals regenerated.

Juveniles. Since there appeared to be a sex-related difference in the success of regeneration, juvenile rabbits of both sexes were ear punched at two weeks of age, in order to avoid the effects of high titers of hormones associated with later sexual develoment. Within this group of animals ten of the 34 ears punched regenerated within the tenure of the experiment, and as

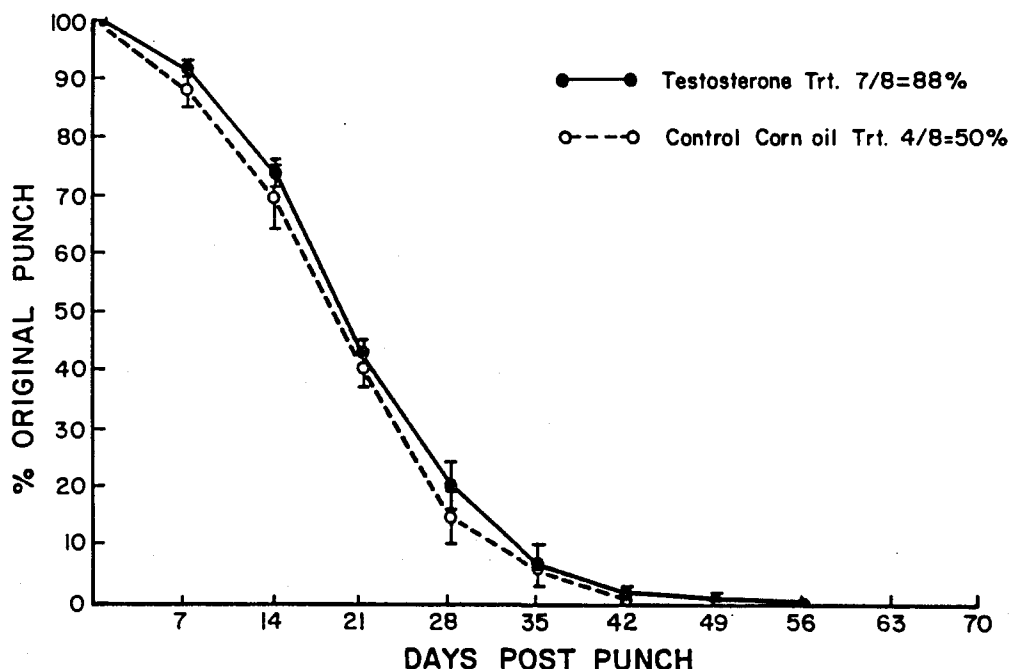


Figure 4. Effect of testosterone administration on ear regeneration. Ratios are number of ears closed/number of ears tested.

the solid line on Figure 5 shows, all were complete by day 56. This graph is misleading in that two of the ten had regenerated by day 16 and six by day 28, independent of sex. This means that the majority of the juveniles completed their tissue replacement one to three weeks earlier than adult rabbits. It must be noted however that due to the small size of the two week old rabbits' ear, holes of only five mm in diameter (1/2 the adult size) could be punched in their ears. Therefore, as the rabbits grew older (ten weeks) their ears were repunched with 0.75 and 1.0 cm holes and no difference was seen in closure rate when the punch was placed on a section of the ear adjacent to the first punch. However, when the punch was made on the site of the original punch the rate of closure increased. The percentage of closures also increased with the second punch, from a success rate of 29% to 67%. As depicted in Figure 5,

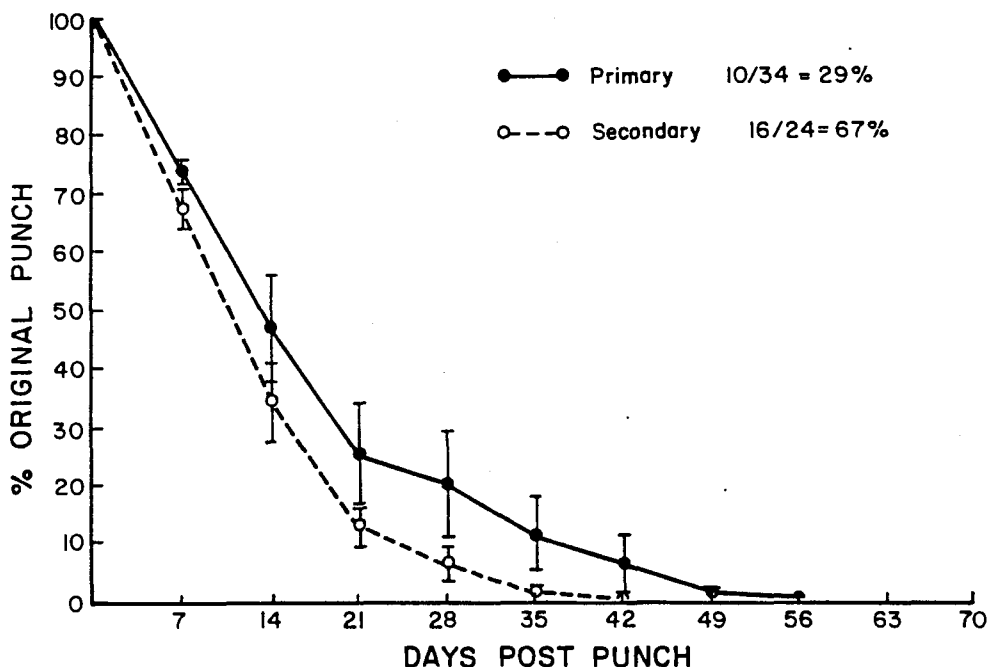


Figure 5. Closure of ear holes in juvenile rabbits after a primary or secondary punch. Ratios are numbers of ears closed/number ears tested.

the secondary punch (i.e. second punch on the primary site) closed one to two weeks earlier than the primary punch (first punch on a site). The photograph of an ear with two punches, one a primary punch and one secondary, is shown in Figure 6.

Secondary punching. Figure 7 shows the results of secondary closure in adult males. The solid line represents the animals that were successful in regeneration with primary punches. Here, five of the six ears that regenerated were complete by day 42. In the secondary punches as noted by the broken line, all six of the successful closures were complete by day 35, with three closing as early as day 21. The minimum of a week's difference may reflect the absence in these animals of the seven day (+) plateau phase of closure noted immediately after punching. Males punched three successive times did not increase their rate the third time over the secondary punch. Females and juveniles also followed a similar pattern. Table I lists the changes in

in thickness of ear tissue once it has been replaced one to three times. There exists a significant increase between primary and secondary punches and very little change with the tertiary.

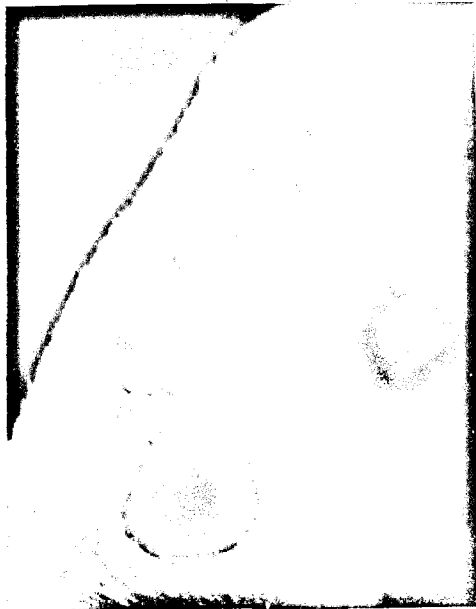


Figure 6. Comparison of ear regeneration between a primary and a secondary punch. See text for details.

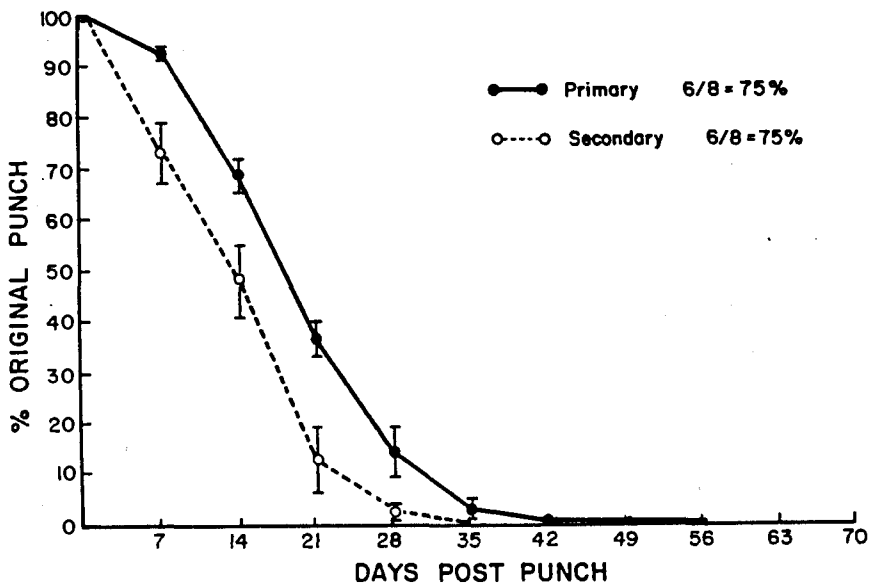


Figure 7. Ear hole closure in adult male rabbits after a primary or secondary punch. Ratios are number of ears closed/number of ears tested.

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SUCCESSIVE PUNCHES	
Animal	
	
Males*	2.40 ± 0.25 (7)
Females	1.43 ± 0.09 (2)
Juveniles	1.57 ± 0.16 (17)

Figure 6. Comparison of ear regeneration between a primary and a secondary punch. See text for details.

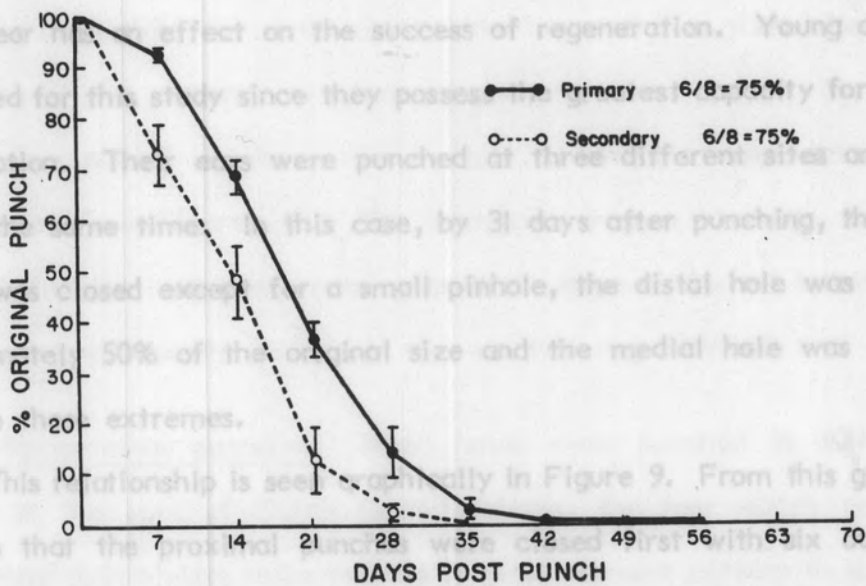


Figure 7. Ear hole closure in adult male rabbits after a primary or secondary punch. Ratios are number of ears closed/number of ears tested.

TABLE I

VARIATIONS IN THICKNESS OF REGENERATED EAR TISSUE WITH
SUCCESSIVE PUNCHES

Animal	Primary	Secondary Punch	Tertiary
Males*	1.05 ± 0.11 (9)**	2.56 ± 0.19 (8)	2.40 ± 0.25 (7)
Females	0.79 ± 0.08 (7)	1.22 ± 0.27 (6)	1.43 ± 0.09 (2)
Juveniles	0.87 ± 0.03 (31)	1.33 ± 0.09 (34)	1.57 ± 0.16 (17)

* over one year of age; ** mean ± S.E. () = n

Location of the punch. If one examines Figure 6, it can be noted that the second punch made on the ear could not always be placed adjacent to the original punch due to the location of the large vessels in the area. Figure 8 shows the results of an experiment to determine if the location of the punch on the ear has an effect on the success of regeneration. Young adult males were used for this study since they possess the greatest capacity for successful regeneration. Their ears were punched at three different sites on the same ear at the same time. In this case, by 31 days after punching, the proximal wound was closed except for a small pinhole, the distal hole was reduced to approximately 50% of the original size and the medial hole was at a state between these extremes.

This relationship is seen graphically in Figure 9. From this graph it can be seen that the proximal punches were closed first with six out of seven complete by day 35. The medial punches were all complete by day 56, with



Figure 8. Regeneration of ear tissue from holes made in different locations on the pinna. Photographed 31 days after punching.

six of the eight complete by day 49. Of the distal punches placed near the periphery of the ear, only one out of eight completed and the graph depicts the mean change of all eight punches. No evidence was found to indicate any stimulatory or inhibitory relationship between concurrent punches placed in any ear. The data yield the same closure kinetics within a given site on the ear regardless of the presence of other punches.

Punches placed on the outer margins of the ear were not capable of regeneration (see Figure 10). The tumescent region normally indicative of blastema function appeared transiently at the edge of the cut but regeneration did not proceed and the defect in the ear was not repaired.

Pigmentation patterns. When holes were punched in different color regions of the ears of Dutch Belted rabbits, the hair which grew on the regenerated skin always reflected the original pigment pattern in six of the six

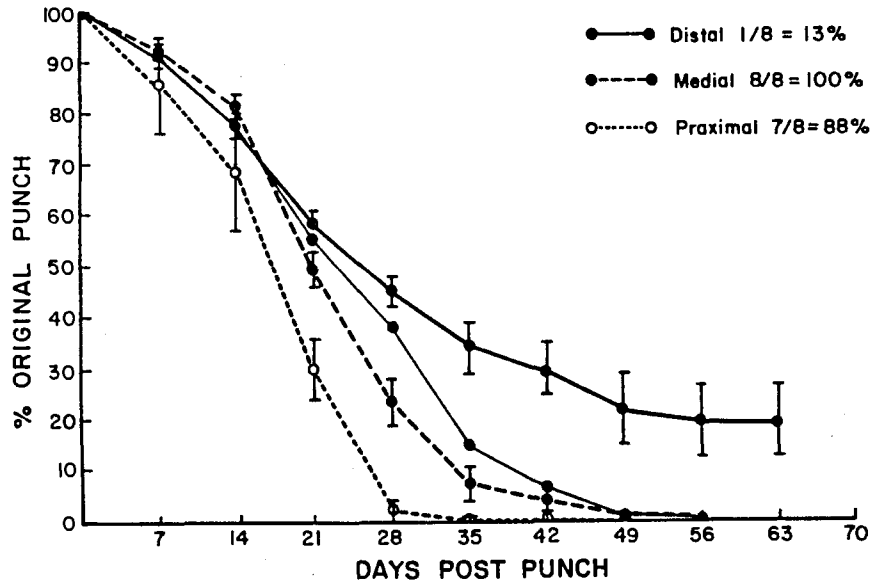


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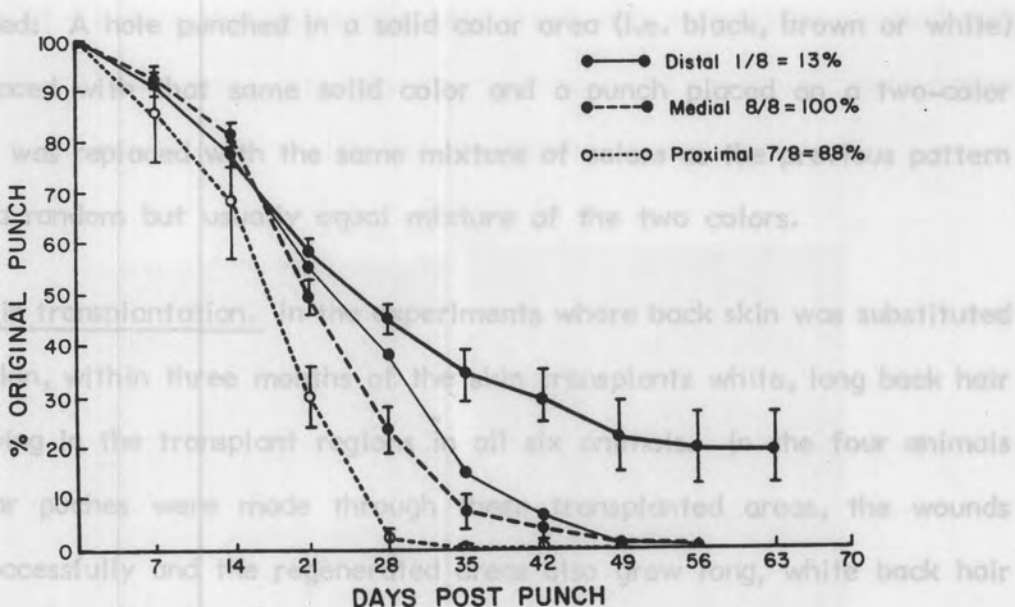
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Closure of ear holes punched in different locations. The single distal hole which closed is shown by the thinner solid line. All the distal holes are represented by the heavy solid line. Ratios are number of ears closed/number of ears tested.



Appearance of regenerated area 4 months after punching a hole where white, long-haired skin had been transplanted from the back to the black, short-haired ear of a Dutch-Belted rabbit. The half circle wound in the posterior edge of the pinna was made at the same time as the other hole.



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ears tested: A hole punched in a solid color area (i.e. black, brown or white) was replaced with that same solid color and a punch placed on a two-color boundary was replaced with the same mixture of colors as the previous pattern or with a random but usually equal mixture of the two colors.

Skin transplantation. In the experiments where back skin was substituted for ear skin, within three months of the skin transplants white, long back hair was growing in the transplant regions in all six animals. In the four animals where ear punches were made through these transplanted areas, the wounds closed successfully and the regenerated areas also grew long, white back hair oriented in the same direction as the graft and easily distinguishable on the black short-haired ears (Figure 10). In Figure 11 the photographs are of the graft rotation experiment in a New Zealand white. 11a shows the tissue the day of the graft with a control punch distal on the ear for comparing the pattern of hair regrowth. 11b shows the appearance of the ear 4 months after the transplant. The hair has been stained with India ink for better visualization of the results. On the distal, normal punch regenerate, the hair is in the original orientation. The scant hair on the proximal graft regenerate is longer than the distal hair, (indicative of back hair) and is at 90 degrees to the normal ear hair orientation.

Toe transplantation. Figure 12 shows positive prints from the X-ray pictures taken of one ear where toes had been transplanted. The results shown in the figure are typical in that no regenerated bone was demonstrable in six separate experiments. Where whole toes had been transplanted, regeneration proceeded normally from the edge of the holes bordered by ear tissue but was generally retarded where the toe tissue was exposed. This usually resulted in an uneven pattern of hole closure, most of the tissue migration being from

the sides of the hole thus giving the healed but still incompletely closed area an elongated appearance. However, the toe tissue did not inhibit regeneration when it had been inserted in the skin pouch before punching.

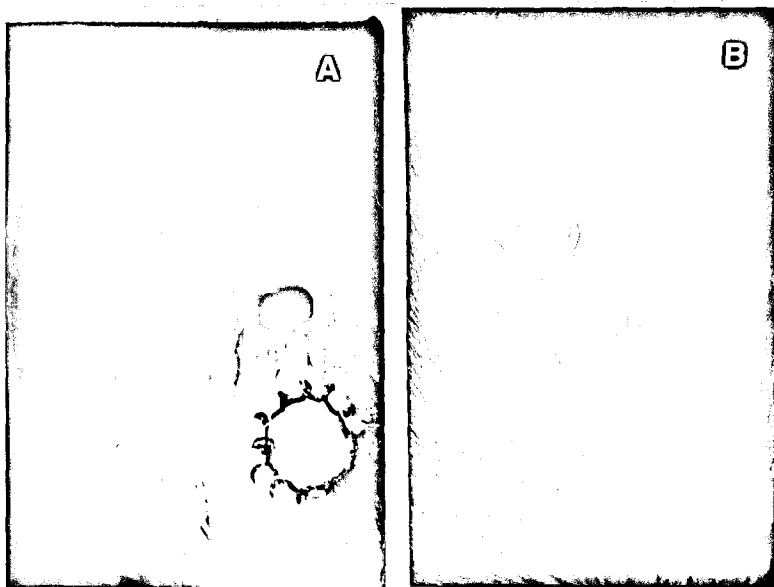


Figure 11. Transplantation of back skin with 90 degree rotation to dorsal ear. (11A) day of surgery, (1) with control punch distally (2) (11B) 3 months after punch through graft. Tissues have regenerated with hair at 90 degrees (1) to the control punch hair (2) or the remaining ear hair. Hair on both regenerates is stained with India ink.

Discussion

With these studies it has been shown that the events associated with the closure of a wound placed in the ear of the rabbit are governed by several factors. In general the data confirm the time requirements for regeneration as reported by earlier workers (Grimes and Goss, 1970; Goss and Grimes, 1972, 1975). However, some other aspects of these studies do not agree with the literature.

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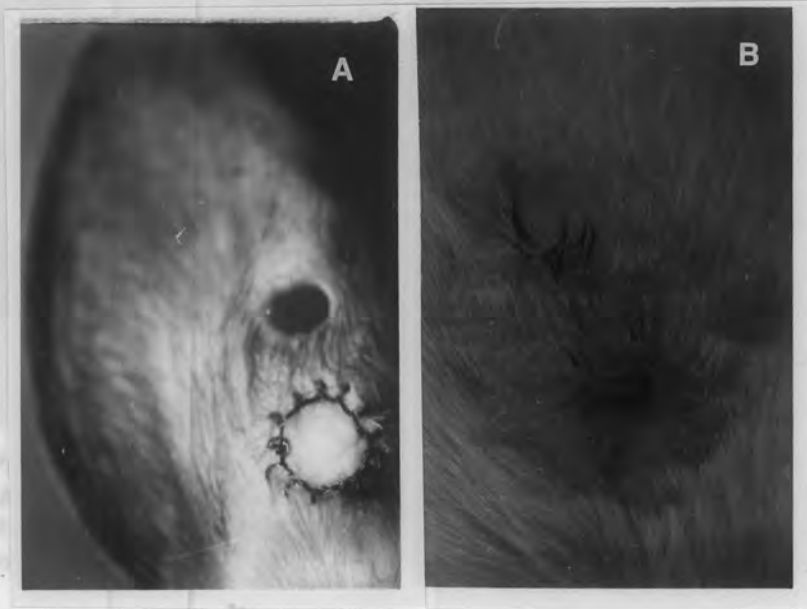


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Discussion

With these studies it has been shown that the events associated with the closure of a wound placed in the ear of the rabbit are governed by several factors. In general the data confirm the time requirements for regeneration as reported by earlier workers (Grimes and Goss, 1970; Goss and Grimes, 1972, 1975). However, some other aspects of these studies do not agree with the literature.

Joseph and Dyson (1966a) with studies on the histological progression of rabbit ear tissue regeneration (RETR) reported that completion of the wound

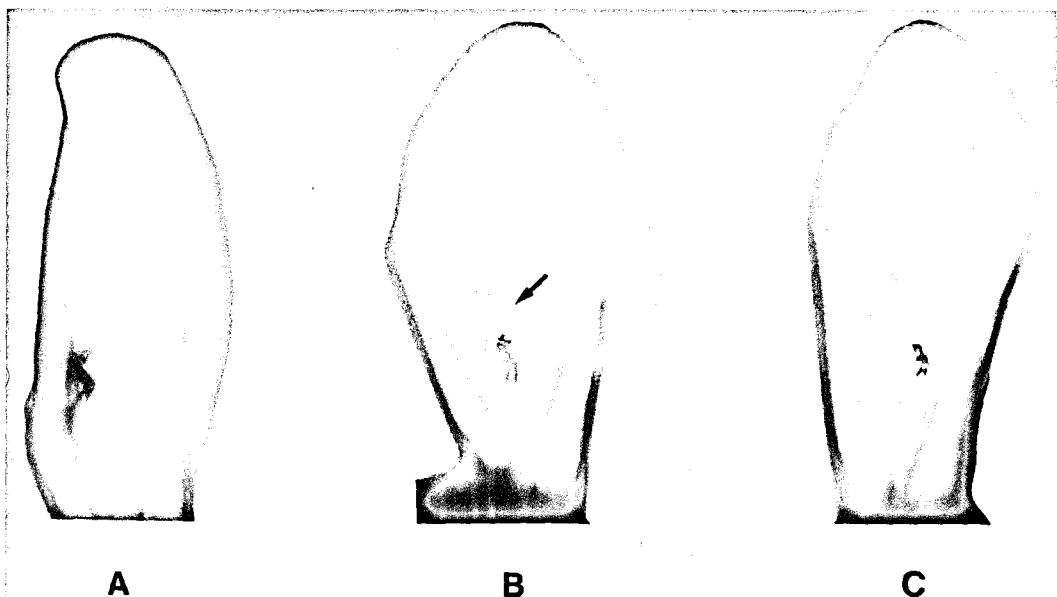


Figure 12. Positive photographs made from X-ray negatives of a rabbit ear. (A) after transplantation of a toe (B) after punching the ear so the edge of the hole cuts through the implanted toe and (C) 2 months later, after ear tissue regeneration has been completed.

closure took over 90 days and they made no mention of animals in their study groups which were unsuccessful in regenerating lost ear tissue. From our initial study we found that 37% of the animals were successful in replacing their lost tissues, and that 95% of those that were successful had done so by day 49. In observations of animals up to one year after punching, if they had not completed the repair by day 60, very little repair could be expected to continue beyond that point. Superficially the blastema appears to reduce in size beginning around the 49th-50th day after punching.

Dyson and Joseph (1968, 1971), in studying the effects of male and female sex hormones on the rate of the regenerative process, claimed faster regeneration in males. They concluded that testosterone induced some enhancement of tissue repair. It is known that large doses of androgens may inhibit production of granulation tissue, yet most indications are that they

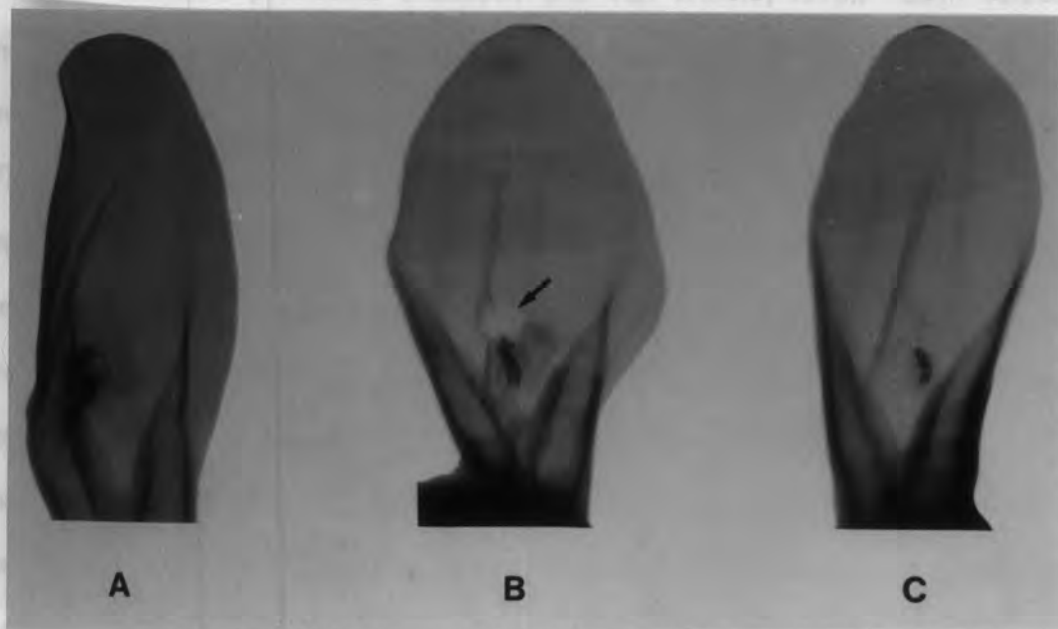


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promote reparative processes (Peacock and Van Winkle, 1976). Both estradiol and progesterone stimulated regenerative growth in females, and only progesterone did so at extremely high doses in the male (Dyson and Joseph, 1971). Our studies show no significant difference in the rate of ear tissue replacement between males and females but the proportion of males which were successful in regeneration during the tenure of our experiments was greater than females (75% and 20%, respectively). In fact, the successful females in our study often completed ear closure as much as a week earlier than the males. Furthermore, when testosterone was administered to ovariectomized females, no increase was seen in the rate of replacement, but again a significant increase in the frequency of successful regeneration (80% compared to 50% in animals given carrier alone). Pregnancy seems to heighten a rabbit's capacity for RETR. However, lactation appears to have no significant effect on RETR different from nonparous animals.

The results of the study with juvenile rabbits further complicate the issue of steroid hormone effects on regeneration. Even though young animals are not under the influence of large titers of hormones associated with sexual development, the duration of repair for the majority of the juveniles within our primary study group was one to three weeks earlier than adult animals. In addition to this difference in rate of closure the juveniles did not experience the approximate one week plateau phase, believed to be the time required for wound repair and to initiate the blastema growth, as do adult animals immediately after punching. This increase in initial closure rate with juveniles may simply reflect the systemic growth phase (i.e. the period of growth all animals undergo to adulthood) that these animals are experiencing, while in adults growth is only occurring normally for tissue repair or maintenance and would most likely require longer to initiate healing.

The second and third punch placed on a previously regenerated site regenerates faster than the first one. This phenomena was seen in both adults and juveniles; with secondary and tertiary punches closing as much as one to two weeks faster than the primary punches. This may indicate that the tissues were in some manner 'primed' for the regeneration process and that the factors are present to initiate or to continue growth and replacement may still be present after an initial punching. Whether these factors (cells?) specifically migrate or diffuse to these areas, or are formed locally in response to the wounding of the tissues is not known.

It seems apparent that there are systemic (e.g. hormonal) as well as local (e.g. position effect) influences over the ability of tissues to regenerate. The hormonal status of the animal may determine whether or not the animal responds but only under the auspices of its own genome since in all groups of animals tested there were those which would not respond under any of the study regimes used. It was also observed that in all experimental groups there was at least one animal whose ears differed in response from each other.

With this study information is presented as to the significant effect of the location of the punch on the pinna regenerative success. Until this time no mention of this phenomenon has existed in the literature. The increased rate of closure and higher frequency of regeneration with punches placed in the proximal regions can offer interesting clues to the factors necessary for regeneration. One particularly obvious difference between the proximal and distal regions of a rabbit's ear is the amount of cartilage and connective tissue present. Goss and Grimes (1972) postulate that the cartilage may serve as a source of blastema cells, induce other tissues to produce a blastema, or act as a mechanical barrier between the two layers of skin preventing healing of the wound by direct contact and thus allowing for regeneration. Their experiments

offered no clue as to what the real role might be but that the cartilage was necessary for regeneration. Differences in the levels of innervation and of vascularity might also account for proximo-distal differences in regenerative ability (Weisbroth, 1974).

An additional factor seems to play a role in the success of regeneration. Holes placed on the perimeter of the pinna, leaving an open edge, were not successful in regeneration regardless of their proximal to distal placement (Figure 10, Page 33). As well as there being a variation in cartilage thickness (i.e. cartilage layer exists throughout the pinna but is thinner at the distal perimeter of the ear) the regenerative process appears to require a complete rim of tissue, a 360° wound with all edges involved in healing. The converging of ear tissue toward the center of the wound may require certain positional information from local interactions between cells and their nearest neighbors which might only be provided by a wound bounded on all sides by tissue (Bryant et al., 1981).

The tissue interactions involved in providing a suitable environment for regeneration are of primary importance in understanding this question of why the ear is capable of regeneration while other tissues are not. Goss and Grimes (1972) studied the interaction of skin and cartilage in this process and found that skin from elsewhere on the body grafted to both sides of the ear could not support regeneration with the subsequent replacement of the cartilage. We replaced only the dorsal ear skin with back skin and found after punching that the tissue did regenerate with the regenerate eventually growing long white hair indicative of back skin. If the epidermis slides over the wound as is seen in other healing wounds (Peacock and Van Winkle, 1976) then we would expect to see hair follicles on the regenerated tissue in both the original orientation and in the rotated orientation. Since we only see the hair on the

regenerates in the orientation of the grafts, we suggest that the epithelium does not simply slide across the wound with contraction in the first phase of regeneration but rather the basal epithelial cells at the wound periphery proliferate and migrate across the wound carrying the morphological as well as spatial characteristics of the strip of epithelium which immediately surrounds the wound. These results are consistent with the observations of Breedis (1954, 1962) on the regeneration of epidermal appendages by differentiation of the migrated epithelium in the rabbit and deer. Punching ears with two color patterns further substantiates the origin of the hair follicles from the tissue immediately adjacent to the wound. The hair pigment pattern replaced on the regenerate was always the same as, or very similar to, the original hair scheme on the ear, whether the punch was placed on a solid color patch or on the boundaries of white and black regions. In view of this replacement of hair color pattern one can suggest that even more complex tissue interactions occur in regeneration, with each site on the wound periphery contributing to regeneration of the lost tissues.

That skin from the back could regenerate when placed in the environment of the ear, encouraged us to investigate whether more complex structures composed of many tissue types could also regenerate under these same conditions. In this context however we were unable to demonstrate that tissues from toes transplanted to the ear could regenerate. Obviously there are limits to the tissue types and complexities which can regenerate in the environment provided by the ear.

Ear punching is simple to perform and easily monitored, both morphologically and physiologically. With the current observations certain optimal factors have been elucidated which will increase the success of

regeneration after punching. These data should facilitate the acceptance of the RETR system as a model for regeneration studies in the mammal.

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EAR TISSUE REGENERATION IN OTHER MAMMALS

Introduction

As ear tissue regeneration is the only good example of epimorphic regeneration in mammals, it is important to know what other species besides the rabbit have this capacity. Goss and Grimes (1975), claimed that the lost ear tissue of sheep and dogs will not regenerate. With references to other investigators, they stated that rabbits are unique as the only mammals capable of regenerating the ear tissues lost in punches. No mention of attempts in any other species, is found in these referenced papers, however. Goss and Grimes (1975) attributed this failure of regeneration in sheep and dogs to the lack of development of epidermal downgrowths, which they believe form as a result of a chondro-epithelial interaction. Carlson (1978b) also implies that rabbits are unique in this capacity to regenerate lost ear tissues although no specific attempts in other animals are cited. In view of the lack of evidence in the literature, fifteen genera of animals normally maintained in laboratory, zoo or agricultural facilities were ear punched to determine what other animals might possess the capability to regenerate lost ear tissues and therefore might provide additional models for research into mammalian regeneration.

A widely accepted method of marking laboratory and agricultural animals for identification is by ear punches placed on the edges of the pinna. The effectiveness of this identification depends on the lost tissue not being replaced. Since the punches or notches are made on the distal edges where the ear contains the least amount of cartilage, the opportunity for successful regeneration is diminished. Not even rabbits will consistently regenerate lost

tissues when punched on these sites (Williams-Boyce, and Daniel, 1980). To avoid this problem, the animals were ear punched on the proximo-medial aspect of the pinna.

To monitor the success of regeneration the ears were repunched after the animal had closed the defect or when the allotted time for observation was passed. Histological preparations were made of both the original punch and the secondary punch, thus our data for the additional species is limited to only two samples per ear (four per animal). Since we had originally used the regeneration of the cartilage layer as a sign of successful regeneration in the rabbit, the histology of the cartilage was used to evaluate the ear tissues for regeneration success in the additional animals. Comparisons were made between distinctive histological characteristics of the rabbit ear tissue regenerates and those of a selected number of the other mammals.

Examples from four orders of mammals were compared with our experimental model, the rabbit of the order Lagomorpha. Representatives of four families of the order Rodentia were selected and mice as laboratory research models, chinchillas as non-laboratory models, and the springhare of Kenya since it occupies the same ecological niche in East Africa as the rabbit occupies in North America. Three families from the order Primates were selected: (1) to compare old and new world monkeys and the marmoset, and (2) as potential clinical models. Three families from the order Artiodactyla were used; cows, pigs, and sheep as agricultural models and the dik-dik as an exotic model. From the order Carnivora, the dog was selected as a potential clinical veterinary model. If available both males and females were punched to determine if the same variation existed between sexes in success of regeneration as observed in our initial studies of the rabbit.

Materials and Methods

Anesthesia was used whenever required to prevent discomfort to the animals and to aid in restraining them but routinely the simple procedure did not necessarily require anesthesia or tranquilization. Rabbits were tranquilized with injections, intramuscular (IM), of Innovar-Vet (Pitman-Moore, Inc.) at a dosage of 0.3 cc/kg body weight. Mice were anesthetized with Avertin 0.1 cc/5g body weight plus 50-100 μ l more. Dogs were anesthetized with Tiopental to effect. Primates were anesthetized with Ketamine hydrochloride at a dosage of 10 mg/kg. The animals were restrained and punches were made on the proximo-medial aspect of the ear between the medial artery and marginal veins with a metal punch (see Chapter II). Depending upon the size of the animals ear the punch size varied from 0.2 to 1.0 cm in diameter. To determine if the location effect seen in the rabbit held true for other animals, chinchillas of both sexes were punched on the distal as well as proximal aspect of the ear.

The rate of ingrowth of the ear tissues was recorded as a percentage of the original wound diameter. Histological preparations of the original punches and punches made after wound closure were fixed in Bouins fixative, dehydrated in alcohol, cleared in xylene, embedded in 60° C paraffin, sectioned at 10-15 μ m, stained with hematoxylin and eosin and photographed.

Results

Success of regeneration. Table 2 is a list of the animals tested, the success of these to close an ear punch-wound with or without any visible regeneration (as accessed by replacement of the lost cartilage), and the duration of the observation, and/or time needed to close and regenerate. Of the animals tested the rabbit remains the most proficient at ear tissue regeneration. The rabbit data are from the initial studies of adult males and

TABLE 2
EAR TISSUE REGENERATION IN MAMMALS

Animal*	Number of Animals Punched (2 ears each)	None	Partial	Closure**			Closure Time***
				Complete/Regeneration			
				Non-Reg	Reg	% Reg	
Rabbit	10 Male	0	6	0	14/20	70%	5-6 Weeks
<u>Oryctolagus cuniculus</u>	9 Female	0	12	0	6/18	33%	5-6 Weeks
Chinchilla	5 Male	0	2	2	6/10	60%	4-11 Months
<u>Chinchilla laniger</u>	5 Female	0	7	1	2/10	20%	6-10 Months
Cow	3 Female	0	0	2	4/6	67%	1.5-2.0 Months
<u>Bovis bovis</u>							
Pig	3 Male	0	1	1	4/6	67%	2-2.5 Months
<u>Sus scrofa</u>	3 Female	0	1	3	2/6	33%	2-2.5 Months
Rat	5 Male	0	0	8	2/10	20%	5 Days
<u>Ratus ratus</u>							
Mouse	5 Male	1	7	1	1/10	10%	3 Weeks
<u>Mus musculus</u>	4 Female	1	0	6	0/7	0	1.5-2 Weeks
Primates							
Marmoset	5 Male	0	9	0	1/10	10%	7 Months
<u>Callithrix jacchus</u>							
Stumptail	2 Male	0	3	0	1/4	25%	5 Months
<u>Macaca arctoides</u> ,	1 Female	0	2	0	0/2	0	5 Months
Afr. Green	3 Female	0	6	0	0/6	0	5 Months
<u>Cercopithecus aethiops</u>							
Rhesus	3 Male	0	6	0	0/6	0	5 Months
<u>Macaca mulatta</u>							
Squirrel	2 Male	0	4	0	0/4	0	5 Months
<u>Saimiri sciureus</u>	1 Female	0	2	0	0/2	0	5 Months
Springhare	2 Male	0	0	1	1/2	10%	4 Months
<u>Pedetes capensis</u>	1 Female	0	1	0	0/1	0	6 Months
Sheep	2 Female	0	0	2	0/2	0	2 Weeks
<u>Ovis aries</u>							
Dog	1 Male	0	0	2	0/2	0	11-15 Days
<u>Canis familiaris</u>	1 Female	0	0	2	0/2	0	11-15 Days
Kirk's Dik-Dik	2 Male	2	0	0	0/2	0	6 Months
<u>Madoqua kirkii</u>							

*Arranged in decreasing order of success, **Tabulated as # of ears, ***Duration of Observation
Non-Reg = no visible cartilage regeneration; Reg = visible cartilage regeneration

females seen in Chapter I. These data are listed so that a comparison could be made with similar data from the additional species. The histology of the normal ear as represented by the chinchilla can be seen in Figure 13. The ear contains a middle layer of elastic cartilage with its thin layer of perichondrium (arrow). On each side of the cartilage is dense connective tissue covered by keratinized dorsal and ventral skin. The primary ear punches of all the animals tested were composed of these same tissues and differed very little histologically.

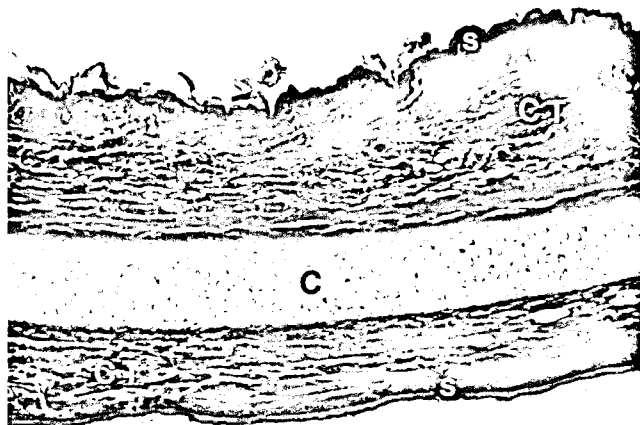


Figure 13. Photograph of a cross section of normal adult chinchilla ear. The ear is essentially a cartilage sandwich with dense connective tissue (CT) between the cartilage (C) and the dorsal and ventral skin (S). The dorsal ear skin is more heavily keratinized than the ventral. L.M. x 51.

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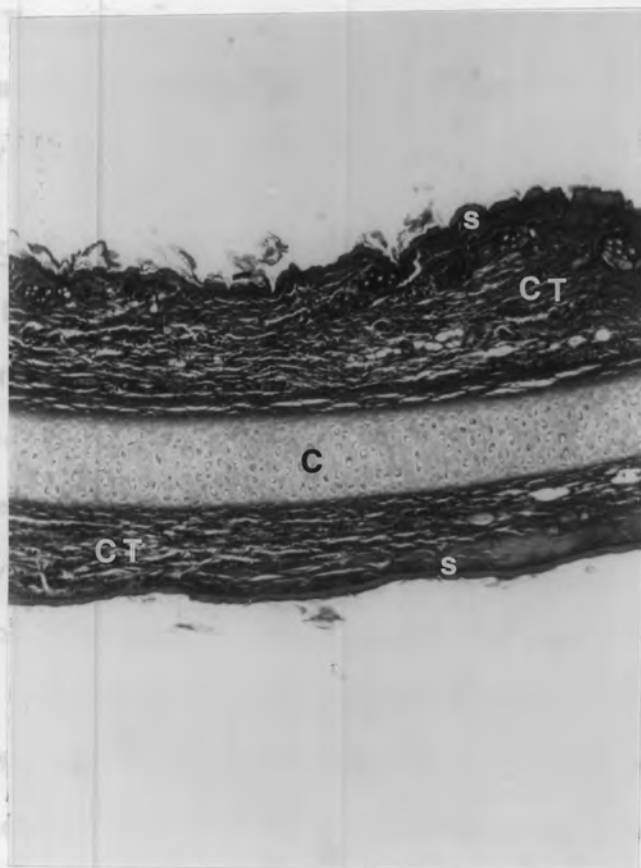


Figure 13. Photograph of a cross section of normal adult chinchilla ear. The ear is essentially a cartilage sandwich with dense connective tissue (CT) between the cartilage (C) and the dorsal and ventral skin (S). The dorsal ear skin is more heavily keratinized than the ventral. L.M. x 51.

From the table one can readily see that wound closure (i.e. of filling in of the defect) is not equivalent to regeneration since there were animals that closed their wounds with no regeneration of the cartilage layer (i.e. Non-reg). In the rabbit, closure is accompanied by continued growth of the tissues until morphogenesis is complete but some individuals of the other mammals tested such as the chinchilla, cow, pig, rat, sheep, mouse, dog, and dik-dik closed their wounds with fibrous scar tissue and showed no signs of cartilagenous regeneration. In a portion of those that closed, thickened regions composed of immature cartilage cells extended distally over the wound were seen histologically. The percentages of animals which closed their wounds and showed these cartilage growth regions range from 10% for the marmoset to 67% for the cow.

Histological observations on the regeneration of the cartilage layer. In

the cow, two different types of cartilage replacement were seen. In one type, cartilage regrowth appears to occur from the perichondrium. In Figure 14, a longitudinal section (i.e. in the horizontal plane of the wound) of the cartilage layer in the regenerating bovine ear can be seen. Mature chondrocytes that have pulled away from their capsules can be seen distal to the wound center within darker staining ground substance (Figure 14, region a). The numerous fibers which are most likely elastic fibers are obvious in the mature tissue at higher magnifications. Young, recently regenerated cartilage is seen near the healing tip of the cartilage layer (Figure 14, region b). The chondrocytes in this layer which are seen to contain 3-5 nucleoli at higher magnifications, are surrounded by a lighter staining matrix. The third layer, region (c), of the regenerating cartilage contains cells which are rounded, contain 4-6 nucleoli (seen at higher magnifications) and are separated by little ground substance.

Such cells are referred to as undifferentiated mesenchymal cells by other authors (Joseph and Dyson, 1966a, b; Vorontsova and Liosner, 1960; Hay, 1959). Seen occasionally between these cells at higher magnifications are characteristic elongate cells which are most likely fibroblasts (Arrow—Figure 14). Fibrous connective tissue containing only fibroblasts occupies the central region of the wound. Often a large blood vessel was observed at the wound center but only when the surrounding cartilage appeared to still be proliferating. This type of regeneration, which appears to be from the perichondrium is very similar to the normal appositional growth of cartilage (Junqueira et al., 1977).



Figure 14. Photograph of a longitudinal section from the cartilage of the regenerating ear of a cow biopsied at 2 months. Region (a), mature cartilage; (b), young cartilage; and (c), 'perichondrial-like' blastema. Arrow—fibroblast-like cell. L.M. x 26.

In Figure 15 is seen an additional type of regeneration in the cow. The cut edge of the mature cartilage is easily visible in the elastic matrix (arrow tips). This layer bears all the characteristics of mature cartilage. At the wound center within thick collagenous strands are seen rounded cells (double arrows) with the mesenchymal-like cell morphology described by Hay (1959).

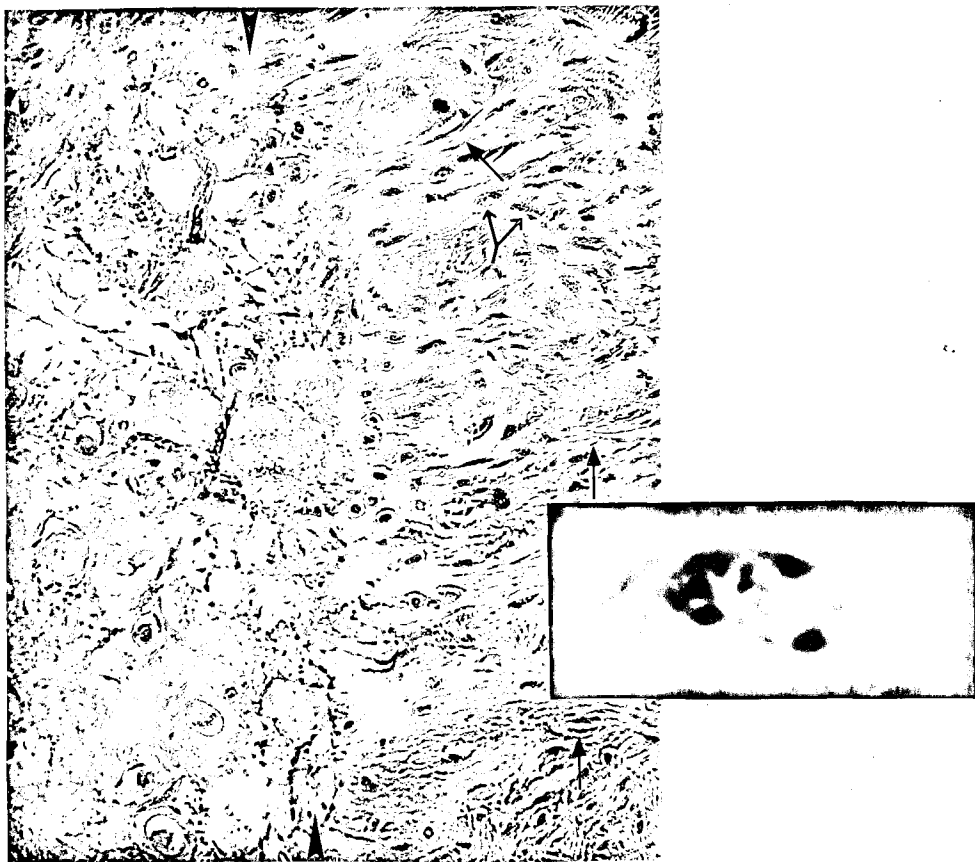


Figure 15. Photograph of a section from the regenerating tissues of a cow's ear. Arrow tips indicate line of biopsy with ear punching. Tissue to the left of tips is mature cartilage with obvious matrix fibers, to the right of the tips, newly formed tissue containing blastema-like cells, collagenous fibers and scattered fibroblasts. Inset, blastema-like cell with rounded nucleus, low cytoplasmic/nuclear ratio, and numerous nucleoli (L.M. x 597). Heavy arrows, collagen bundles. L.M. x 65.

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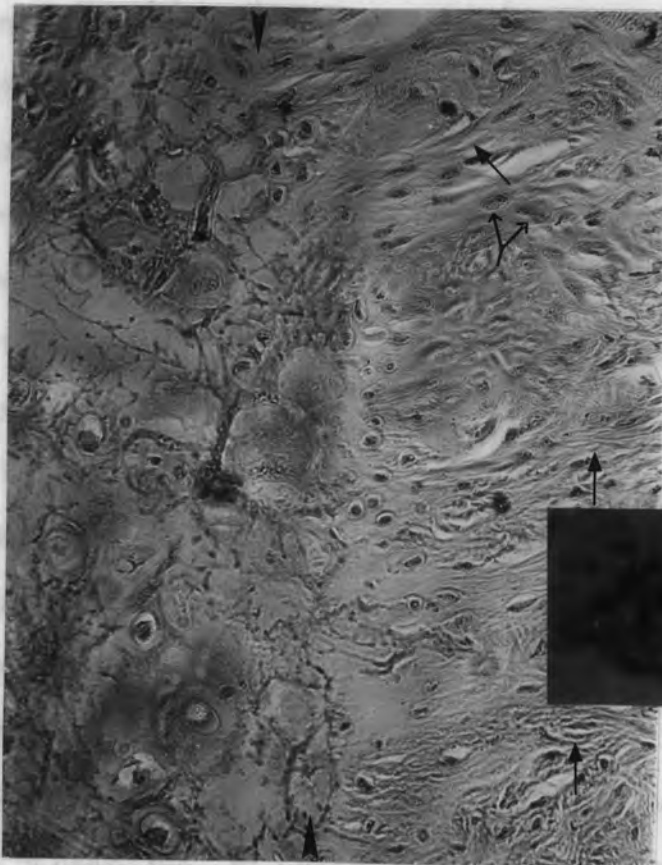


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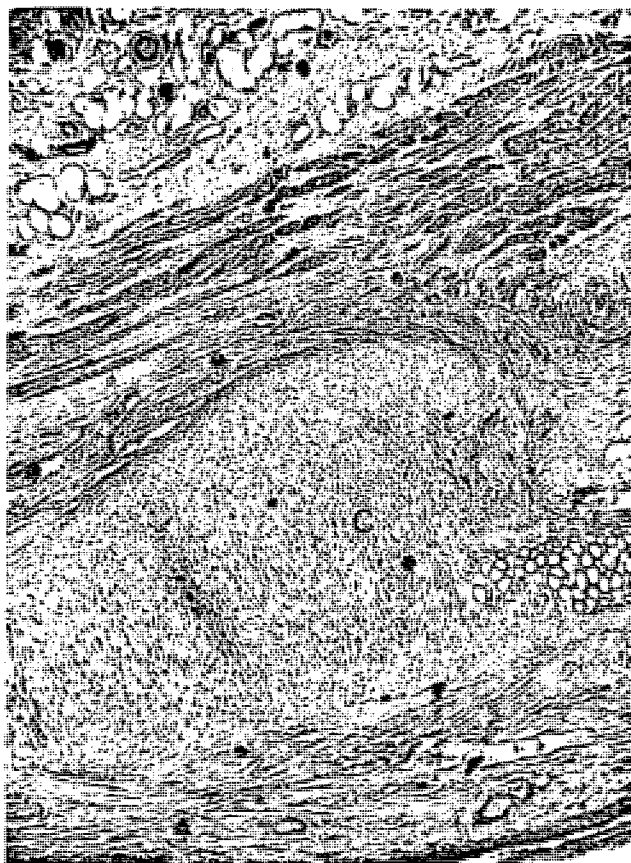
These 'blastema' cells (small arrow) are seen to aggregate along the edge of the mature cartilage. In some locations cells with an intermediate morphology, more like that of immature cartilage, are seen at higher magnifications (Rhodin, 1974). Even though the ear punch wounds in the cow were closed by two months, in neither of the two methods of regenerating the lost cartilage was regeneration of the cartilage layer complete in this amount of time.

Sections of the 'cartilage proliferation' region in the rabbit and chinchilla are seen in Figure 16. We refer to this region as the 'cone' region of the aggregating immature cartilage cells. The cone appears to arise from a proliferation of the perichondrial region and is made up of chondroblasts which will form the chondrocytes of the new cartilage. At the periphery of the cartilage cone is a layer very similar to the perichondrium found in the normal ear cartilage and it is continuous with the perichondrium of the mature cartilage. Chondrogenesis occurs at the cut edge of the old sheet of cartilage.

The apparent proliferation of the perichondrium after punching is also visible in Figure 17. In a section from chinchilla ear, undamaged mature cartilage is seen in the most distal aspect of the wound. At the dorsal aspect of the mature cartilage layer the perichondrium appears to have proliferated from the stimulation of the wounding of the adjacent tissues.

A perichondrial cone was not seen in sections where regeneration failed to occur (Figure 18). In Figure 18, a section from an animal showing no cartilage regeneration, the cut edge of the cartilage appears unstimulated; no apparent perichondrial accumulation of immature chondrocytes is seen, yet small numbers of blastema cells are visible at higher magnifications in the connective tissue at the tip of the undamaged cartilage layer. The matrix at the tip of the cartilage is primarily collagen bundles (identified by Trichrome

a



b

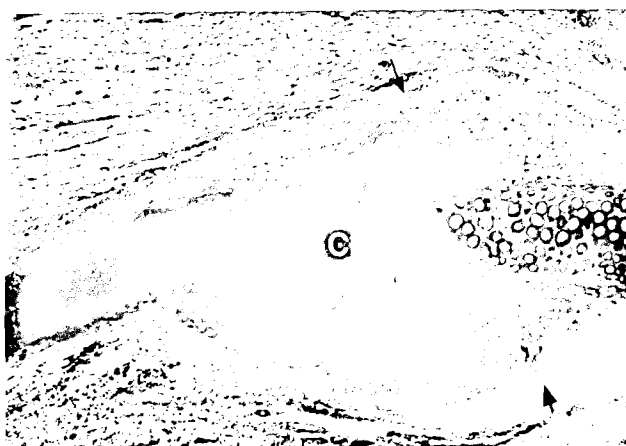


Figure 16. Photographs of the cartilage 'cone' (c) regions of the regenerating ear tissue of the chinchilla 10 months after punching (a) and the rabbit (b). (a) L.M. x 26, (b) L.M. x 22. The old cartilage sheet with its obvious lacunae is seen at the right of each section. In (b) note the perichondrial layer at the periphery of the immature cartilage (arrows).

a



b

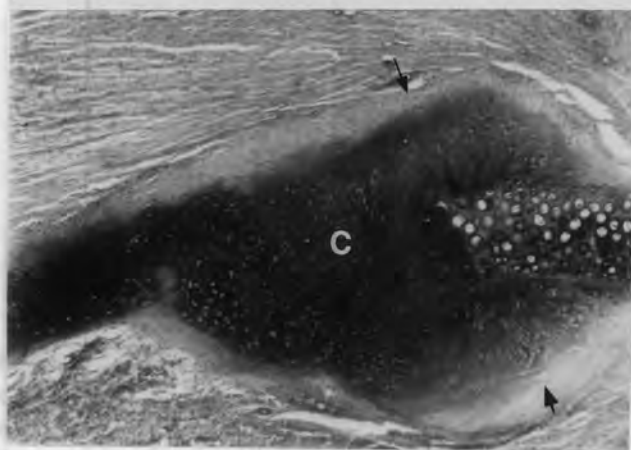


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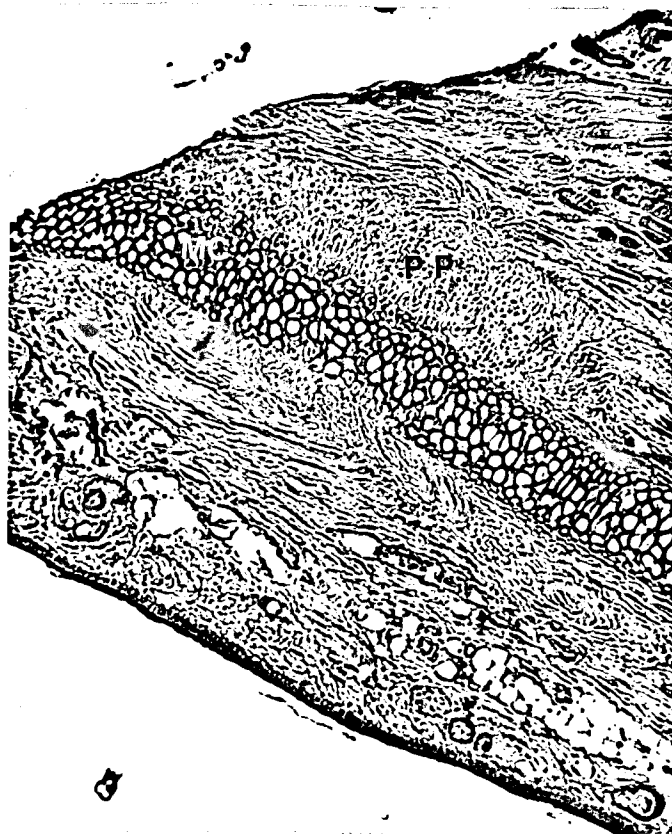


Figure 17. Photograph of a cross section of undamaged chinchilla ear tissue adjacent to the regenerating ear tissues. The perichondrium appears to have proliferated which is most likely due to the stimulation of damage in the nearby tissues. PP = 'perichondrial proliferation' region. MC = mature cartilage. L.M. x 26.



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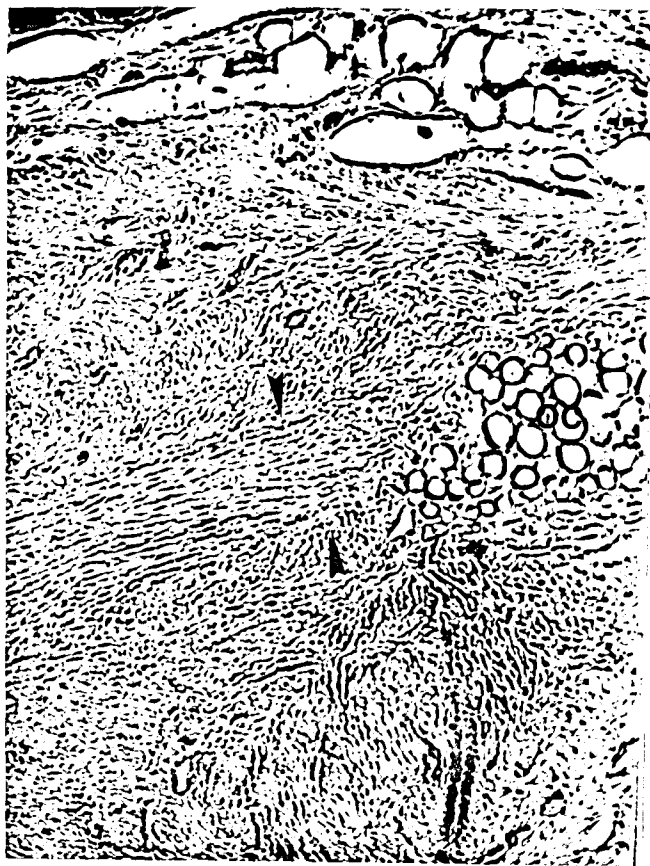


Figure 18. Photograph of a chinchilla ear 10 months after punching. No accumulation of immature chondrocytes is seen at the cut edge of the old cartilage (OC). Bundles of collagen are seen at the tip of the mature collagen in line with the direction of growth (arrows). L.M. x 65.

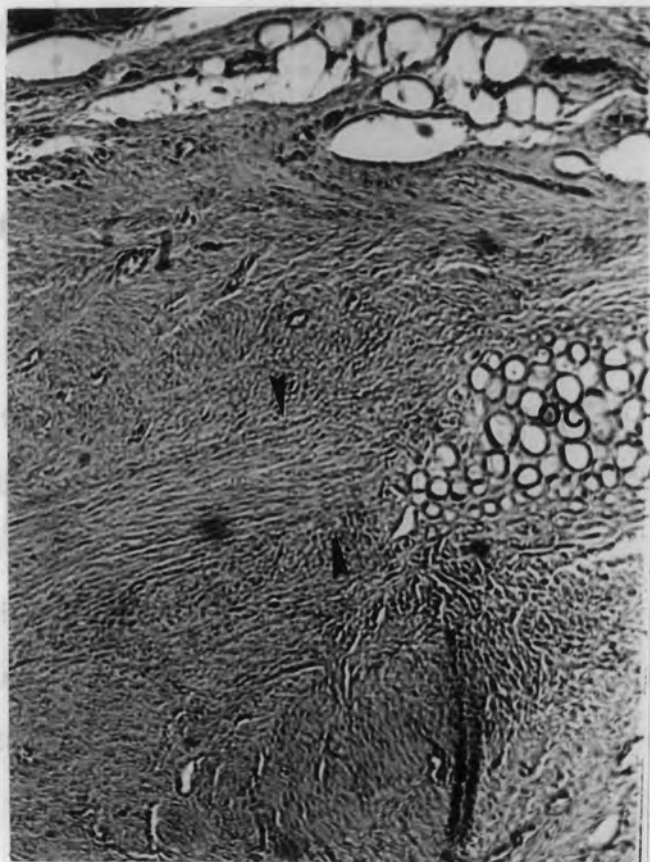


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staining) and these are in line with the direction of growth. This same observation of collagen in linear bundles is seen in the rabbit. In the cow, shown in Figure 15, page 49 the bundles are short, numerous and randomly arranged. The orientation of these collagen bundles may provide scaffolding for cell migration but conversely the random arrangement of these may impede cellular migration.

Other variations in the regenerating cartilage layer were seen. In a rabbit, biopsied at 265 days after punching, not only was the original layer of cartilage replaced but in the greatly thickened layer of perichondrium which surrounded it was found an extensive network of trabecular bone (Figure 19). This section resembles sections of immature bone in the developing mammalian embryo (Ham and Leeson, 1961; Reith and Ross, 1977). The bone is still surrounded by cartilage which on H&E staining is basophilic whereas the bone is acidophilic (Figure 19a). In this sample the perichondrial layer was greatly thickened surrounding the regenerated cartilage and it can be hypothesized that it is within this thickened layer that the bone developed. The bone trabeculae enclose spaces which are similar to both haversian canals and marrow cavities (Figure 19b). Osteoblasts with their darkly staining cytoplasm are arranged along the surface of the trabeculae. In some regions the bone is similar to compact bone with a more regular matrix and smaller haversian canals. A higher magnification of one of these spaces containing a blood vessel is seen in Figure 20.

In addition to the distinctive characteristics of the regenerating cartilage layer, an unusual feature of the wound epithelium was noted. In a section taken from the regenerating ear tissues of a stump-tail monkey, epidermal downgrowths were seen similar to those reported by Goss and Grimes (1975).

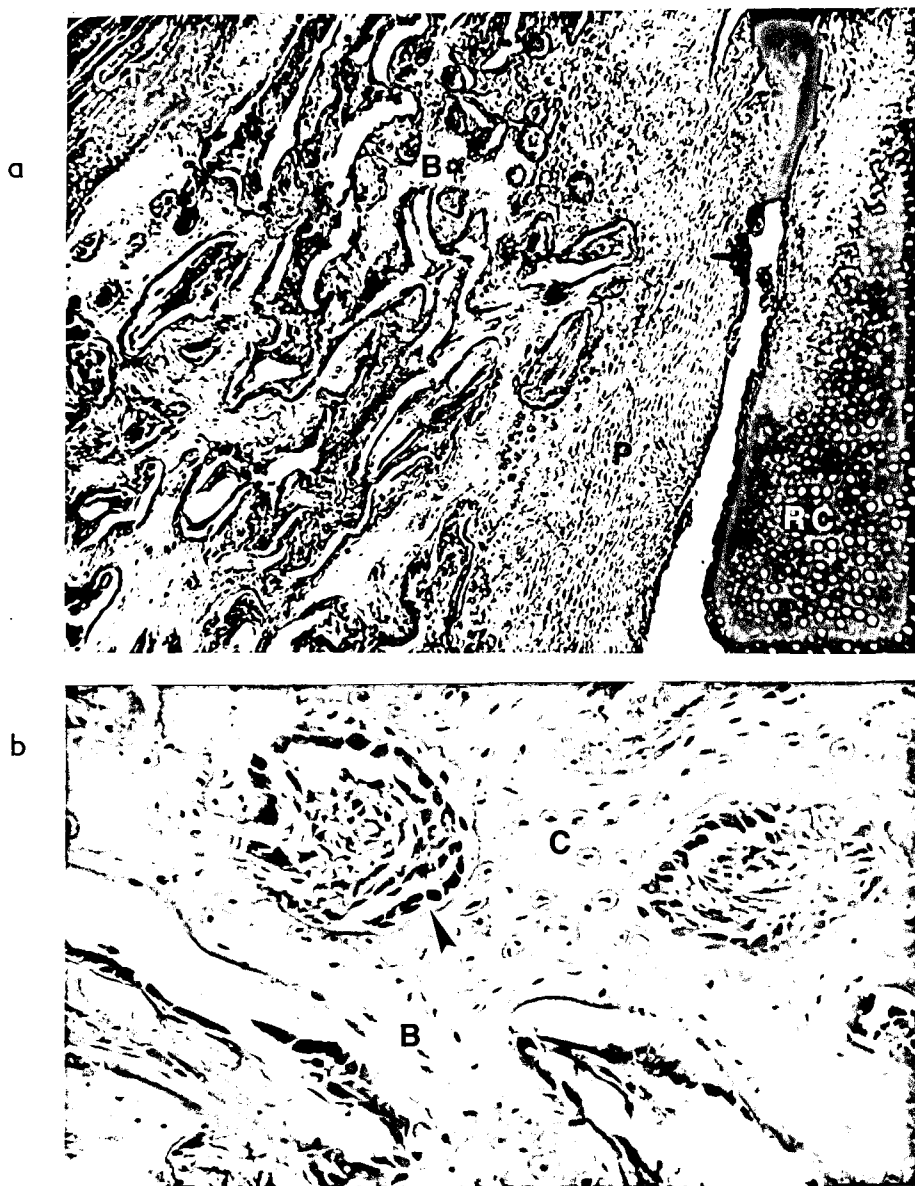


Figure 19. Photographs of sections from the regenerating ear tissues of a rabbit 256 days post punch. In (a), 4 regions of connective tissue are seen: Upper left dense connective tissue (CT) middle bone (B), perichondrium (P), regenerated cartilages (RC). In (b) both bone (B) and cartilage (C) are seen: Bone is found immediately surrounding the marrow cavities and cartilage is found in between. The marrow cavities are lined with chondroblasts (arrow) (a)—L.M. x 15, (b)—L.M. x 113.

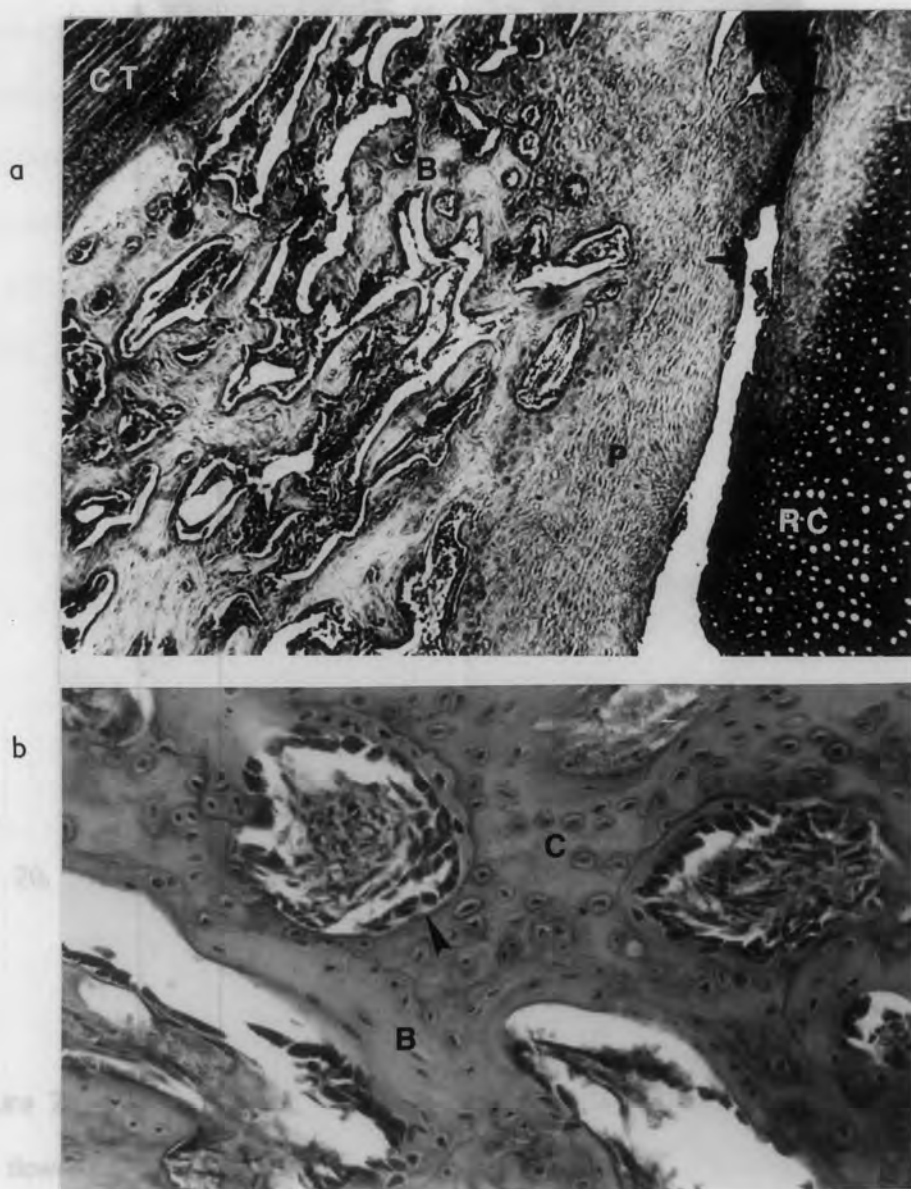


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Figure 20. Photograph of a section of bone from the regenerating ear tissues of a rabbit 265 days post punching. The heart shaped structure appears to be a marrow cavity lined with osteoblasts (arrow). A blood vessel (BV) containing blood cells is seen at the top left of the cavity. L.M. x 340.

In Figure 21 the downgrowths can be seen at the dermis- epidermis interface. These downgrowths or 'fingers' are not as large as those seen in the rabbit but these animals are also not as successful at regeneration. The tissue under the epidermis contains large quantities of randomly arranged collagenous fibers.

Location of the punch. To test the effect of location of punch, on success of regeneration, adult chinchillas (Chinchilla laniger) of both sexes were punched on the distal and proximal portions of the ear. The animals were unsuccessful in replacing tissues lost in punches placed on the outer

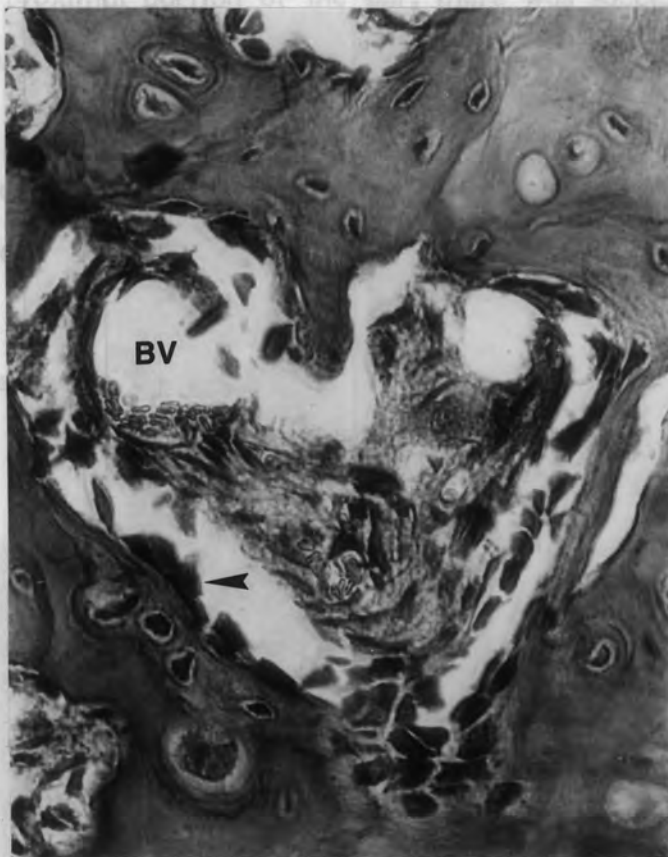


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margin of the distal surfaces of the ear. However when the punches were placed on the proximal portion of the ear, there was successful regeneration. Closure of a 0.5 cm hole took an average of 10 months. The same variation in success of regeneration previously observed in the rabbit, Figure 3, page 26 (Williams-Boyce and Daniel, 1980; Joseph and Dyson, 1965, 1966a; and Dyson and Joseph, 1968), and pig, was seen in the chinchilla, Figure 22. Males were 75%, 60% and 67% successful, for the rabbit, chinchilla and pig, respectively. Females of the same species were 33%, 20% and 33% successful.



Figure 21. Photograph of a section from the leading edge of the epithelium in the regenerating ear tissues of a stump-tail monkey. Epidermal downgrowths (ED) are seen around the periphery of the wound. L.M. x 65.

Discussion

Ear tissue regeneration can no longer be considered a phenomenon limited to the rabbit. Rabbits remain the most successful of all mammals tested yet

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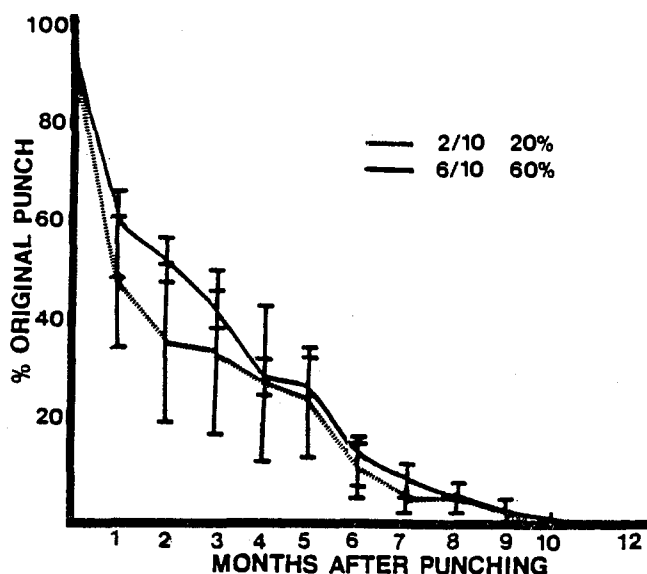


Figure 22. Duration of closure after ear punching in adult chinchillas. Broken line-females, solid line-males. Ratios are number of ears closing/number of ears tested.

the chinchilla, cow, pig, rat, mouse, marmoset, stump-tail monkey and the springhare have shown limited success by the methods used in this study.

We feel that one factor in the reported lack of success with other animals has been due to the location of the punch. As exhibited by the chinchilla, when punches were placed on the distal portion of the pinna there were no successful closings or regeneration of the tissues lost. Yet when punches were placed on the proximal portions as close to the head as possible there was both closure and regeneration. This phenomenon of location effect on the success of regeneration was first demonstrated by punching three different sites simultaneously on the rabbit ear (Williams-Boyce and Daniel, 1980). Proximal punches closed faster and more frequently than medial or distal punches.

No experimental evidence has been obtained to explain why the proximal portion of the pinna is more successful than the distal. Hypotheses that the better vascularization in this region and the thickness of the cartilage layer

may both contribute to the increased success of the proximal punch have been made (Williams-Boyce and Daniel, 1980). Goss and Grimes (1972) showed that regeneration of the ear tissues was dependent upon the cartilagenous sheet and its interaction with the wound epidermis: If the cartilage is removed before a punch is made, regeneration does not occur. Grimes (1974a) also noted that in the absence of cartilage, ingrowth of the tissues stopped and the holes enlarged somewhat at the time when chondrogenesis normally begins at the wound perimeter.

The elastic fibers of the ear cartilage are most dense in and near the perichondrium, and more scattered in the cartilage proper. In thin ears the ratio of perichondrium to true cartilage is higher than in thicker ears (Joseph and Dyson, 1966b). Even though it has not been proven experimentally, one could suggest that thin ears are more elastic than thick ones. Thus when the ear is punched on its edges or on the distal portion where the cartilage is thinner, the elastic perichondrium may cause the cartilage to retract and actually increase the size of the defect. In our preliminary studies on the rabbit, when we punched the distal portions of the ear we often observed for the first week an increase in the size of the hole over the size of the original punch. No attempts were made to determine if these animals were less successful than others in regeneration, but we hypothesize that they would be. This increase in the size of the defect could prevent the initial interactions between the epidermis and the underlying tissues which is vital to regeneration.

By selectively irradiating either the cartilagenous sheet or the remaining ear tissues without the cartilage layer, Grimes (1974b) found that irradiated ears containing unirradiated cartilage regenerated, but the reverse was not true; irradiated cartilage in unirradiated ears did not support regeneration of

the cartilage layer. Apparently, normal cartilage and its adherent connective tissues (perichondrium) are important sources of cells for the regenerate.

In the appositional growth of cartilage, cells of the perichondrium multiply and differentiate into chondrocytes which are incorporated into the existing cartilage. During growth, superficial areas of cartilage show transitions between chondrocytes and cells resembling the fibroblasts (fibroblastic stem cells) of the perichondrium (Junqueira et al., 1977). Also when cartilage fractures, cells from the perichondrium invade the fractured area and generate new cartilage (Junqueira et al., 1977). In the normal processes of growth and repair therefore the perichondrium serves as the source of new cartilage cells. It is plausible to assume that the perichondrium may serve a similar function in regeneration. The cells of the perichondrium are capable of proliferation, migration, and differentiation, all requirements of the cells needed to replace the lost cartilage and perhaps serve as the precursors of other blastema cells.

The role of these perichondrial derived cells in the production of other types of tissues is not known. Hay (1959) with EM studies on the regenerating salamander limb, noted the reversion of muscle and cartilage cells to a common basophilic cell type with all the ultrastructural characteristics of embryonic mesenchymal cells indistinguishable from one another. Steen (1968) showed that limb chondrocytes of the axolotl are intrinsically stable as to cell type. Some grafts of both triploid and H^3 -thymidine labeled chondrocytes give rise to morphologically dedifferentiated blastema cells which almost exclusively differentiate into chondrocytes. In more recent studies with chondrocytes in culture, these cells were seen to lose their distinctive chondrocyte morphology and become more fibroblastic in appearance. Concomitant with the altered morphology, these cells stopped producing cartilage type collagen, Type II, and produced the type fibroblasts produce, Type I (Benya et al., 1977; Uitto, 1979).

The sections from the chinchilla and rabbit show an active region of chondrogenesis at the tip of the old cartilage sheet. The importance of this cartilage sheet to successful regeneration is not entirely known. However from other evidence present in the literature and from the present histological observations we hypothesize that the perichondrium may serve as an active source if not the primary source of blastema cells; or that cartilage regeneration in the ear is from both the blastema and by tissue proliferation of the perichondrium. The finding of a thickened region of the perichondrium, not the mature cartilage, in the undamaged tissues at the periphery of the wound in the chinchilla (Figure 16, page 52) lends support to this hypothesis. Rabbit ears whose cartilage is surgically removed leaving the perichondrium primarily intact through the cartilage excised region will regenerate the cartilage from proliferation of the perichondrial fibroblasts and their subsequent differentiation into chondroblasts (Skoog et al., 1972; Skoog and Johansson, 1976; and Wasteson and Ohlson, 1977). These studies also revealed that cartilage devoid of perichondrium will not regenerate and, biochemically, the newly formed perichondrial chondrocytes will form the extracellular matrix component, chondroitin sulfate, by a metabolic pathway similar to that seen in embryonic development.

The finding of bone in one of the older regenerated rabbit ear tissue samples is particularly exciting and is important in two ways. One is the possibility that a number of differentiated ear cells of the adult organism are capable of redifferentiating not only into the lost tissues of the ear but are even capable of using part of the genome they have never expressed by forming a tissue that was never there originally. The finding of these bone tissues is supportive of the theory that ear tissue cells can dedifferentiate since no bone 'stem' cells should exist in the ear. However it could also be suggested that

the fibroblastic stem cells of the perichondrium may form osteoblasts instead of forming chondroblasts (i.e. the conversion of one type of connective tissue to another).

Few examples of spontaneous bone formation exist. Under pathologic conditions in post-natal life tiny pieces of bone may develop in the scars of severe wounds, the tonsils, kidneys or other severely traumatized tissue (Ham and Leeson, 1961). Bone nodules are occasionally found in mammals or in Achilles tendons subjected to trauma or ischemia (Carlson, 1978a); complete bones are found in regenerated newt limb from which the bones are removed prior to amputation (Weiss, 1925; Thornton, 1938); and mixtures of highly differentiated tissues such as hair, teeth, bone and vestiges of other organs are often found in teratocarcinomas and teratomas (Carlson, 1981). Bones have been stimulated to develop in the abdominal muscles of guinea pigs by a bone morphogenetic protein extracted from guinea pig diaphyses (Urist et al., 1972). This protein is believed to be complexed with collagen or to be a derivative of collagen.

From these examples it was suggested that the tissues of the damaged muscle, the limb, and the tumor had dedifferentiated and redifferentiated to form types of cells other than those from which they are derived. We now suggest that the same may be said for the regenerating rabbit ear tissues. The second significance of this find is that the regeneration event in the ear is more complex than that of simple wound healing. Foreign tissue formation never occurs in simple wounds (Peacock and Van Winkle, 1976), but it is found infrequently in severe wounds, tumors, and regenerated tissues.

These studies on a variety of laboratory, zoo and agricultural animals show that the phenomenon of replacement of tissues lost in an ear punch is common to other mammals as well as the rabbit although the rabbit appears

the most proficient in this process. We suggest that the cartilage may be replaced by both epimorphic regeneration, through the formation of a blastema as originally hypothesized by Markelova (Vorontsora & Liozner, 1960), and tissue regeneration, from the perichondrium since animals without the cone of cartilage (perichondrial cone) did not regenerate lost ear tissue. The finding of bone tissue is suggestive evidence that some dedifferentiation or cross differentiation (perichondrial stem cell to osteoblast) may occur. These studies have altered existing concepts relating to tissue regeneration; one by showing that a variety of mammals are capable of ear tissue regeneration, and secondly that the replacement is entirely epimorphic appears to be only partially correct.

COLLAGEN METABOLISM IN REGENERATING EAR TISSUES

Introduction

During growth and development, from embryo to senescence, the organism undergoes remodeling, radical and rapid early in life, more subtle and slowly with increasing age. Reproducibility of form and the repetitive pattern of morphologic changes demand great precision of timing coupled with localization of synthesis, orientation of tissue constituents, and ordered removal of structural elements. The phenomenon of tissue regeneration in the adult i.e. the redevelopment of form and function in an organism which has long since passed through the ordered processes of embryonic growth, may be restricted by an environment of highly differentiated cells. The process of synthesis, orientation, and organization of structural elements such as fibrous proteins is of ultimate importance in regeneration. Removal of such structural elements is of equal importance since organized growth is obviously not a simple matter of accretion. Dismantling of tissue components must be finely controlled, both temporally and spatially, to permit growth, change, and repair without impeding function. Failure of this regulation may lead to malformation or altered physiology.

In this study our aim was to focus on a single, well characterized tissue element, namely collagen, known to be involved in remodeling processes throughout life and compare its metabolism in regenerating and nonregenerating tissues to those seen in the embryo. In the mammal, repair occurs primarily by fibrous tissue proliferation rather than by regeneration of the original anatomical structures. The mechanical properties of normal connective tissue

and of repair tissue, in particular, are due primarily to fibrous proteins of which collagen is the most important.

Collagen is widely distributed throughout the animal kingdom and within the vertebrate organism where, as the structural framework of most tissues, it may account for as much as 30% of the total body protein and in repair tissue, collagen may amount to more than 50% of the total protein (Peacock and Van Winkle, 1976). During wound healing as epidermis migrates over the wound, fibroblasts which are scattered throughout the connective tissue of the injured dermis, migrate towards the wound area in response to the injury, and after arriving increase their number by cell division and begin to secrete collagen. By the time the scab falls off, the scar, with its high content of collagen fibers has been formed (Mattson, 1976). Collagen is involved in nearly all remodeling processes and is found in large quantities in certain tumors (Moro and Smith, 1977).

Healing by fibrous tissue replacement can be both beneficial and harmful, depending on the location of the injury and the tissue being replaced. In bone repair, for instance, deposition of a collagen matrix and its subsequent calcification restore the original structure and return the original function. In contrast, repair of damaged peripheral nerves by fibrous tissue growth can interfere with regeneration of axons and thus prevent restoration of function. Frequently, an overproduction of fibrous tissue, or more specifically changes in the nature and organization of collagen, may lead to functional impairment even though damaged tissue is functionally repaired, such as in tendon repair or in intraabdominal healing where fibrous adhesions may form.

Collagen is a triple-helical molecule made up of three component alpha chains. At least four distinct collagens are found in vertebrate tissues (Miller, 1976). These collagen types, which have some degree of tissue specificity,

differ from each other in their alpha chain composition. The most thoroughly studied collagen contains two polypeptide chains (alpha components) that are chemically identical ($\alpha 1$) while the third ($\alpha 2$), although basically similar, is sufficiently different to permit separation by carboxymethylcellulose chromatography (Piez, 1963). This collagen bears the heteromorphic chain composition in a 2:1 ratio, ($\alpha 1(I)$)₂ $\alpha 2(I)$, is designated Type I, and is the primary collagen in bone, mature dermis, tendon, ligaments, cornea and dentin (Chung and Miller, 1974).

In 1969, a second collagen, Type II, was derived from chick cartilage. It was composed of three identical chains, designated $\alpha 1(II)$ chains since they differed from the $\alpha 1$ chains of Type I (Miller and Matukas, 1969). The predominance of collagen molecules with the chain composition ($\alpha 1(II)$)₃ has been established for several cartilagenous structures in a number of species (Trelstad et al., 1976; Miller, 1971). Type II is also found in the embryonic corneal epithelium, the tissue known to produce the collagenous component of the primary corneal stroma (Linsenmayer et al., 1977), the embryonic chick neural retina tissue in vitro (Smith et al., 1976), the notochord (Smith et al., 1976) and high concentrations of Type II have been found in a transplantable rat chondrosarcoma.

In 1971, a third alpha chain was discovered in infant dermis and subsequently in aorta, parts of the lung, human placenta, uterine leiomyomas in large quantities and skin in small quantities (Bailey and Robins, 1972; Chung and Miller, 1974; Epstein, 1974). In skin, this Type III collagen is present in high concentrations in embryonic dermis and the amount diminishes after birth. Type III collagen contains three unique alpha chains $\alpha 1(III)$.

Type III is also found in inflammatory responses and in abnormal wound healing, for example, in dermal granulation tissue under acute inflammation by

turpentine injection or chronic inflammation by sponge implant and in hypertrophic scars, some ten to fifteen years old (Bailey et al., 1975). In normal healing, small amounts of Type III collagen may be found but the collagen changes with time to young skin collagen. Type I. In contrast, hypertrophic scars fail to follow these time related changes in normal skin, retaining embryonic characteristics of producing large amounts of Type III collaen. Type III is the predominate collagen in embryonic skin but generally normal scar tissue does not form an embryonic type collagen (Shuttleworth et al., 1975).

The newest discovered collagen, Type IV, contains three identical alpha chains different from those of Types I, II, or III's chains. It is found in basement membranes, Descemet's membrane, and the anterior lens capsule (Peacock and Van Winkle, 1976).

In summary, four types of collagen have been identified that appear to be tissue specific. Types II, III and IV are trimers of $\alpha 1(II)$, $\alpha 1(III)$ and $\alpha 1(IV)$ respectively. Type III collagen, though observed in some adult tissues, appears to be found in greatest amounts in embryonic tissues.

In addition to the genetically distinct types of collagen, another molecular form of collagen, $\alpha 1(I)$ -Trimer or Type I-Trimer has been isolated from cultured cloned chick chondrocytes grown in the presence of 5-bromo-deoxyuridine (Mayne et al., 1975). The $\alpha 1(I)$ -Trimer consists of three polypeptides which are genetically identical with $\alpha 1$ chains of Type I collagen. The $\alpha 1(I)$ -Trimer collagen was also isolated from a virus-induced tumor and its synthesis was observed in cultures of cells derived from such tumors (Little et al., 1977; Moro and Smith, 1977). The synthesis of $\alpha 1(I)$ -Trimer molecules has also been observed in cultures of chemically transformed rat liver epithelial cells (Smith and Niles, 1980), and in lathyritic chick embryo tendons and calvaria (Jiminez

et al., 1977). It has been suggested that $\alpha 1(I)$ -Trimer molecules may represent errors in the assembly of collagen polypeptides under tissue culture conditions or in malignant tumors, and these molecules may not be present in normal connective tissue. Little and Church (1977) suggest that Trimer collagen represents an embryonic collagen type and cultured fetal epithelial amniotic fluid cells with a mesenchymal-like morphology have been found to produce Trimer (Crouch and Bornstein, 1978).

Studies of tissues undergoing rapid cellular transformation via either a remodeling process as in growth or with tissue repair after wounding have shown a dramatic resorption of collagen. Extracts of these tissues do not yield an enzyme capable of hydrolysing native collagen (Mandl, 1961; Woessner, 1965; Lapiere and Gross, 1963), however, collagenolytic activity has been detected in the medium of cultured tissues such as tadpole tail, gills, and gut, mammalian uterus, bone, and skin wounds (Gross and Lapiere, 1962; Gross et al., 1963; Walker et al., 1964; Grillo and Gross, 1967; Eisen et al., 1968). This collagenolytic enzyme is synthesized de novo from living cells and accumulates in the culture chamber to detectable levels during the period of incubation.

Adult cells are often immobilized in a collagenous network, whereas cells that demonstrate properties associated with more embryonic states appear much more free to move. The degree to which collagenase activity is causal to the formation of blastemal cells is an intriguing question. The idea that the breakdown of the extracellular matrix is crucial to formation of the blastema from the freed (dedifferentiated cells from the stump) cells was first voiced by Butler and Puckett in 1940. An increase in a number of hydrolytic enzymes are seen in the amputation stump of the newt in the early phases of regeneration; yet none of these proteases are capable of digesting collagen

(review Schmidt, 1968). Grillo et al., (1968) saw callogenolytic activity in the regenerating forelimbs of Triturus during reaneration, but not in tissues proximal to the wound. Nagai et al., (1966) isolated a collagenolytic enzyme from tissue cultures of tadpole tail fin and back skin but not from the skin of mature frogs. Gross and Lapiere (1962) observed that only skin, gut and gill of tadpoles have relatively strong collagenolytic potency, i.e. those same tissues which undergo the most radical remodeling during metamorphosis. This evidence is consistent with the likelihood that this enzyme is physiologically significant in metamorphosis.

Not only is the amount of fibrillar collagen changed during development, repair and regeneration but the biochemical identity of the collagen also changes. A number of embryonic tissues produce collagens whose synthesis are of a transitory nature; for example, the $\alpha(1)_3$ chain of embryonic skin (Miller et al., 1971), the neural tube (Trelstad et al., 1973) and the vertebral bodies (Linsenmayer et al., 1973a). The changes seen in collagen production by cartilage may be indicative of its state of differentiation since one of the generally accepted criteria for differentiated cartilage is the synthesis of the cartilage specific collagen, Type II. During embryogenesis of the long bones and vertebrae of the chick temporal and spatial transitions from 'undifferentiated' mesenchyme, through cartilage, to bone matrix occur with concomitant changes in the type of collagen produced (Linsenmayer et al., 1973b, 1973c). Temporal as well as spatial transitions are seen; i.e. stage 23-24 leg mesenchyme produces predominately Type I but with the subsequent development of long bones, the diaphysis produces Type I, while the cartilaginous epiphysis produces Type II.

During blastema formation chondrocytes in the regenerating limbs of salamanders cease their synthesis of detectable levels of cartilage type collagen,

Type II, and only produce Type I (Linsenmayer and Smith, 1976). Hay (1973) noted, from observations in situ on the regenerating larval amphibian finger that virtually the whole blastema is derived from cartilage (which constituted almost all the mesenchymal cells present before amputation).

A switching was seen in the type of collagen synthesized from Type II to a mixture of Type I and the Type I-Trimer in cultures of cloned chick chondrocytes (Mayne et al., 1976). This same switching in collagen biosynthesis has been observed in cultures of rabbit articular chondrocytes (Layman et al., 1972), human embryonic chondrocytes obtained from fetal bone rudiments (Mayne et al. in Mayne et al., 1976) and chick chondrocytes grown in dialyzed embryo extract (Mayne et al., 1976).

The same temporal changes in collagen type seen in developing tissues, tissues in culture and regenerating tissues are also seen in other matrix components during regeneration and development. Hasty et al. (1981) observed in the rabbit ear regeneration system that position of sulfation of the major glucosaminoglycan (GAG) deposited in cartilage matrix, chondroitin sulfate, changed during the regeneration process. The normal ear and the regenerate during epithelialization and blastema cell proliferation produce primarily chondroitin-4-sulfate whereas regenerates with differentiating cartilage produce more chondroitin-6-sulfate. This shift in sulfation position may mimic the development of fetal rabbit cartilage Mathews (1967). A similar increase in chondroitin-6-sulfate was seen in perichondrial cartilage regenerates of the rabbit (Skoog et al., 1972; Wasteson and Ohlssen, 1977) and in embryonic chick epiphyseal cartilages between 10 and 19 days of development (Robinson and Dorfman, 1969).

Hyaluronate, another ubiquitous extracellular macromolecule has been shown to influence development (Hasty et al., 1981). High levels are seen in

early stages of developing, regenerating, remodeling, and healing tissues. Little is found in mature cartilage and cornea matrix but it is the predominant GAG produced during the morphogenetic stages of development. It has also been seen to block formation of cartilage-like aggregates in cultured chick embryo somite cells (Toole, 1973). Thus while hyaluronate synthesis is correlated with the morphogenetic phase of tissue formation, hyaluronidase activity is associated with the cytodifferentiation phase, e.g. in the regenerating newt limb, hyaluronic acid was found to be high in the dedifferentiating tissues but low in the outgrowth regions where proliferation dominated (Toole and Gross, 1971). These same relationships between hyaluronate and hyaluronidase are seen in the developing chick limb, in chondrogenesis of the chick vertebral column, and in the development of the cornea (Hasty et al., 1981).

The ubiquitous extracellular protein, collagen has proven to be a particularly interesting model for studies on morphogenesis at the molecular level. Collagen is readily isolated, purified, and characterized, both in the solid state and in solution. Under physiologic conditions this protein is remarkably resistant to the known proteolytic enzymes with the exception of its specific proteolytic enzyme, collagenase. Collagen is therefore a near ideal guide to a specific enzyme system operating on a particular structural element during morphogenesis since collagenase degrades only collagen. With our histological observations on the formation of the blastema in the rabbit ear an increase in matrix fibrous proteins which stained specifically for collagen were observed. This accumulation of collagen is not seen in the blastema formed on the amphibian stump after amputation (Schmidt and Jasch, 1971), even though treatment of the blastema with collagenase removes most of the extracellular matrix of the blastema (Schmidt, 1970).

Our studies involve both the production of collagen in the regenerating tissues and its regulation via collagenase. This interplay of synthesis and degradation of structural elements so important in embryonic development may be intimately involved in enabling a tissue to regenerate or preventing it from doing so. We hypothesize that collagen levels will change during the regeneration process from an increase of the early stages to a decrease during differentiation, and that these changes may be due to a collagenolytic enzyme.

Additionally, since most collagens are tissue specific, by looking at the various types of collagens produced by the ear we hope to gain insight into the cell type transitions required for dedifferentiation and redifferentiation. As described in the introduction, Hay (1959) and Hay and Fischman (1961) saw cartilage and muscle cells dedifferentiate into cells unrecognizable from each other. Since cartilage produces a very specific collagen type, it would be interesting to see if these cells have dedifferentiated biochemically as well as morphologically. If the new chondrocytes simply arise from old we would expect to see the continued production of Type II cartilage, if however the new cartilage cells arise from either the perichondrium, as suggested in Chapter III, or from a fibroblastic-mesenchymal type cell we would see Type I collagen.

Finally the changes seen in type of collagen produced by cultured embryonic cells encouraged us to compare the ear regeneration system with embryogenesis, asking if biochemical changes might occur in the regenerating ear collagen as they do in developing tissues and if they do, could they have a role in the development of the ear tissues with regeneration.

In view of the evidence presented in this review it is hypothesized that the regenerating ear tissues are more similar to embryonic tissues than to the tissues comprising a wound. To test this hypothesis collagen was used as a biochemical marker of the developmental changes which occur in regeneration.

Materials and Methods

Collagen typing: collagen extraction. Collagen was extracted by a modification of the methods of Chung and Miller (1974) and Bailey et al., (1975). Samples of ear tissue from the punch-regenerating areas of 2-3 ears from each weekly time point of the regeneration process were pooled, minced, and then extracted for 6 days at 4° C in 100 volumes of 1.0 M NaCl 0.05 M Tris, pH 7.5, with stirring. The tissues were then placed in 0.5 M acetic acid and homogenized in an Oster blender at 4° C. The extracts were centrifuged at 25,000 x g, 0° C for 1 hour, lipid was removed by filtering through glass wool, centrifuged again and filtered through Whatman #1 filters. Some of the samples were extracted by limited pepsin digestion. The extracts were placed in 0.5 M acetic acid and treated with 100 µg/ml pepsin for 6 hr at 15° C. The pH was then adjusted to 8.0 with NaOH to inactivate the pepsin. The solubilized collagen was purified by reprecipitation against 5% NaCl. The clear but viscous solution was then dialyzed against 0.01 M Na₂HPO₄ to precipitate collagen, the collagen was collected by centrifugation at 10,000 x g for 20 minutes, redissolved in 0.5 M acetic acid, dry NaCl added to 10%, precipitated by recentrifugation, resuspended in 0.1 M acetic acid, lyophilized, and stored at 0° C until needed. Two to three pools of tissue were analyzed for each weekly time point, for a total of 23 pools.

Collagen typing: differential salt precipitation. The collagen samples were subjected to differential salt precipitation according to the methods of Chung and Miller (1974) and Trelstad et al., (1976). The entire lyophilized collagen sample from each weekly time point was redissolved at 4° C in 1.0 M NaCl, 0.05 M PO₄ pH 7.5 and the NaCl concentration was increased in

successive steps by 0.1 M over the range 1.0 to 4.0 M until the collagen precipitated by stirring in the cold. Each precipitate was then recovered by centrifugation at 10,000 x g for 20 minutes, dialyzed against distilled water in Spectrapor #1 tubing, lyophilized and stored frozen.

Collagen typing: CM-cellulose chromatography. Separation of the various collagen alpha chains was performed according to the methods of Piez et al., (1963) on 2-3 pools of each weekly time point during regeneration. Lyophilized collagen samples were redissolved at a concentration of 10-25 mg/ml in 0.02 M sodium acetate buffer pH 4.8, containing 1.0 M urea and denatured by warming to 45° C for 30 minutes. They were then applied to a carboxymethylcellulose column 0.5 cm x 9 cm, preequilibrated with deaerated buffer at 42° C, and washed with two column volumes of the starting buffer. The proteins were eluted at 40-42° C with a linear gradient of sodium acetate 0.4 M, urea 1 M, pH 4.8 starting buffer and a limiting buffer of 0.04 M pH 4.8 plus 0.08 M NaCl, total gradient volume 250 ml with a flow rate of 50 ml/hr. After cooling to room temperature the absorbance of each fraction was read at 230 nm. The fractions under each peak were retained, concentrated in Spectrapor #1 to 1 ml volume, dialyzed against distilled water (DH₂O), lyophilized and stored frozen. The column was standardized with rabbit ear skin Type I, and ear cartilage Type II isolated by the above methods.

Collagen typing: electrophoresis. Extracted collagen from both the chromatography columns and differential salt precipitation were separated electrophoretically. The lyophilized samples were solubilized in 0.02 M sodium acetate buffer, pH 4.8 and desalted by dialysis against sample buffer. The amount of protein in each sample was determined by the method of Lowry et al. (1951) 100-200 µg protein was incubated with 1% SDS-1% mercaptoethanol

(except where indicated, i.e. the mobilities of $\alpha 1$ and $\alpha 2$ chains of collagen are the same in the presence or absence of mercaptoethanol, but the migration of Type III changes) for 10 minutes at 100° C. They were transferred to wells of a 7.5% SDS polyacrylamide gel (bisacrylamide/acrylamide, 0.8/30) topped with 3% acrylamide stacking gel, and the electrophoresis was carried out for 2.5 hours at room temperature. Both gel and electrode buffers were Tris 50 mM; boric acid 30 mM at pH 8.5 (Koenig et al, 1970). The gel was stained with Coomassie Blue (0.5% solution in 40% methanol:acetic acid 1 M) for 16 hours and then destained in 40:10 methanol:acetic acid until clear.

Hydroxyproline analysis for total collagen. Homogenates of ear tissue from 2-7 ears at each weekly interval in the regeneration process were analyzed for collagen content primarily by the method of Edwards and O'Brien (1980) and Kwasigroch and Neubert (1980). Biopsies taken at weekly intervals after day of the punch were weighed, minced, and homogenized in 6 N HCl at 1 ml/10 mg tissue with a motor driven teflon pestle for ten minutes on high. The protein concentration of the homogenates were determined by the method of Lowry et al., (1951). Four equal samples (0.2-2 ml) were taken of the homogenate, placed in capped tubes and autoclaved at 120° C for 4-24 hours to complete hydrolysis. The hydrolysates were then placed in a 60° C vacuum oven with dessicant and a NaOH trap until dry. The samples were rehydrated with assay buffer (133 g citric acid 1 H₂O, 32 ml glacial acetic acid, 320 g sodium acetate 3H₂O, 91 g sodium hydroxide, 800 ml n-propanol, taken to 3000 ml with DH₂O, pH adjusted to 6.0-6.5 with 0.2 M NaOH, to total volume of 4000 ml, 1 ml toluene added as a preservative). Four aliquots were removed from each sample and assayed for hydroxyproline. The samples were analyzed directly against standards (Kwasigroch and Neubert, 1980), or by the standard

addition technique which eliminates the need for correction of matrix effects between the specimen and standard (Edwards and O'Brien, 1980). Standards were added to 1/2 of the samples, then freshly prepared Chloramine-T reagent (1.141 g chloramine-T to 100 ml DH_2O) was added, mixed well, and allowed to react for 20-25 minutes at room temperature. The aldehyde perchloric reagent (15 g p-dimethyl-amino-benzaldehyde, 60 ml n-propanol, 26 ml perchloric acid at 70% to 100 ml with DH_2O) was prepared and added to the samples. This mixture was allowed to react for 15 minutes at 60°C at which time the red chromophore develops. The samples and standards were cooled to room temperature and the absorbance read within 3 hours at 550nm. Levels of hydroxyproline were determined by linear regression and standard conversion (Appendix I). The amounts were converted to collagen assuming a standard 12.5% hydroxyproline content for the hydrolyzed collagens. Data were graphed as amount of collagen per gram wet weight sample, or per gram protein.

Collagenase levels. Collagenase levels were measured indirectly by the collagen gel substrate method by calculating the increasing area of lysis around explants in culture (Grillo et al., 1968; Oxlund and Andreassen, 1980). To prepare the sterile collagen substrates, lyophilized collagen extracted in mass from whole rabbit ears using the above neutral salt extraction method was placed into solution, 1%, in 0.16 M phosphate buffer, pH 7.6 by stirring at 40°C for 24 hours. The solution was cleared by centrifugation at $75,000 \times g$ for 1 hour at 40°C . The samples were then dialyzed in Spectropor #1 membrane against 0.005 M phosphate, 0.14 M NaCl pH 7.4 and 0.75 cc of the sterile collagen solution was placed into the center well of a Falcon tissue culture dish. The plates were placed in a CO_2 incubator (37°C) for 3 hours to gel-precipitate the collagen. The gels were then bathed with warm sterile Ham's

F-10 medium without serum containing 150 units/ml penicillin-streptomycin. Fungizone 0.37 mcg/ml for 4 hours to remove any salts in the gel and to replace them with isotonic media. Any free fluid was poured off prior to adding the tissue sections.

Rabbit ears were repunched at weekly intervals. Sections of the punches were immediately rinsed 3X in the complete culture medium and placed cut edge down on the collagen substrate. The cultures were allowed to develop for 3 days (checked daily against a dark background with reflected light) using 0 day as the control. Scores were assigned on the amount of collagen cleared, ranging from 0.5-4.0 depending on the extent of lysis. Controls of bacterial collagenase were used to determine the function of the assay. Additional controls included tissues punched from the margin of the pinna and back wound tissues removed after 1 cm punches of back skin down to the fascia were removed from anesthetized animals.

Results

Collagen typing: differential salt precipitation With differential salt extraction most of the collagen types can be isolated: Type III precipitates at 1.5 M NaCl, Type I at 2.2-2.4 M, Type II and Type I-Trimer at 0.4 M (Chung and Miller, 1974; Bailey et al., 1975; Narayanan and Page, 1976). In Table 3 are listed the major collagen types found during ear regeneration.

In non-regenerating ear tissues or day 0, the principle types of collagen are distinctly segregated. Type I in the skin and loose connective tissues, Type II in cartilage. After punching, these relationships change. The unusual type collagen, Type III, is found in the wound epidermis of the regenerating tissue by day 14 and no more is detectable after day 28. Type I is consistently found in the skin and underlying connective tissues and by day 28 the appearance of

another unusual collagen, the Type I-Trimer is seen in the blastema. By day 49 chondrogenesis is rapidly occurring in the core of the blastema with the appearance of the cartilage-specific collagen, Type II. The blastema still produces Type I and in some samples a small amount of Type I-Trimer is still found. By one year the tissues contain the same types of collagen they did before the tissues were punched, cartilage contains Type II, skin and connective tissue contain Type I. For internal controls, sections of skin excised from the backs of animals were allowed to heal and sampled at one month, only Type I collagen was seen. Type I was also the only collagen found in the tissues from a 42 day marginal ear punch, as seen in Figure 10, page 33.

TABLE 3
COLLAGEN TYPES IN REGENERATING EAR TISSUES

ANALYZED BY DIFFERENTIAL SALT PRECIPITATION					
Day of Sampling	Tissues Sampled	1.5 m (Type III)	2.4 m (Type I)	4.0 m (Type II or α13)*	NACE
0 day	skin c.t.	-	+	-	
	cartilage	-	-	+(C)	
14 day	skin	+	+	-	
	blastema	-	+	-	
28 day	skin	***	+	-	
	blastema	-	+	+(T)	
35 day	skin	-	+	+(T)**	
	blastema	-	+	+(T)	
	core blastema	-	+	+(T)	
49 day	skin	-	+	+(T)**	
	c.t. blastema	-	+	+(T)**	
	blastema core	-	-	+(C)	
1 year	skin	-	+	-	
	cartilage	-	-	+	
1 month	back dermal	-	+	-	
	wound	-	+	-	
42 day	marginal ear punch	-	+	-	

(-) = no precipitate, (+) = precipitate, (*) = results substantiated by CM-cellulose chromatography, (**) = small precipitate (C) = cartilage Type II, (T) = α13-Trimer

Collagen typing: CM-cellulose chromatography. To verify the results obtained from the differential salt fractionation, the samples were applied to CM-cellulose columns. Figure 23 shows the chromatography profile of the standard collagens isolated from a homogenate of all ear tissues except cartilage (solid line) and from cartilage only (broken line). These same profiles were obtained with or without differential salt extraction which illustrates the homogeneity of the preparations. The ratio of $\alpha_1:\alpha_2$ is 1.9:1 for the skin and connective tissue, the ratio expected for Type I cartilage. The $\alpha_1:\alpha_2$ ratio for the cartilage precipitate is 12:1, the ratio expected for Type II cartilage. These results are in agreement with the differential salt precipitation data.

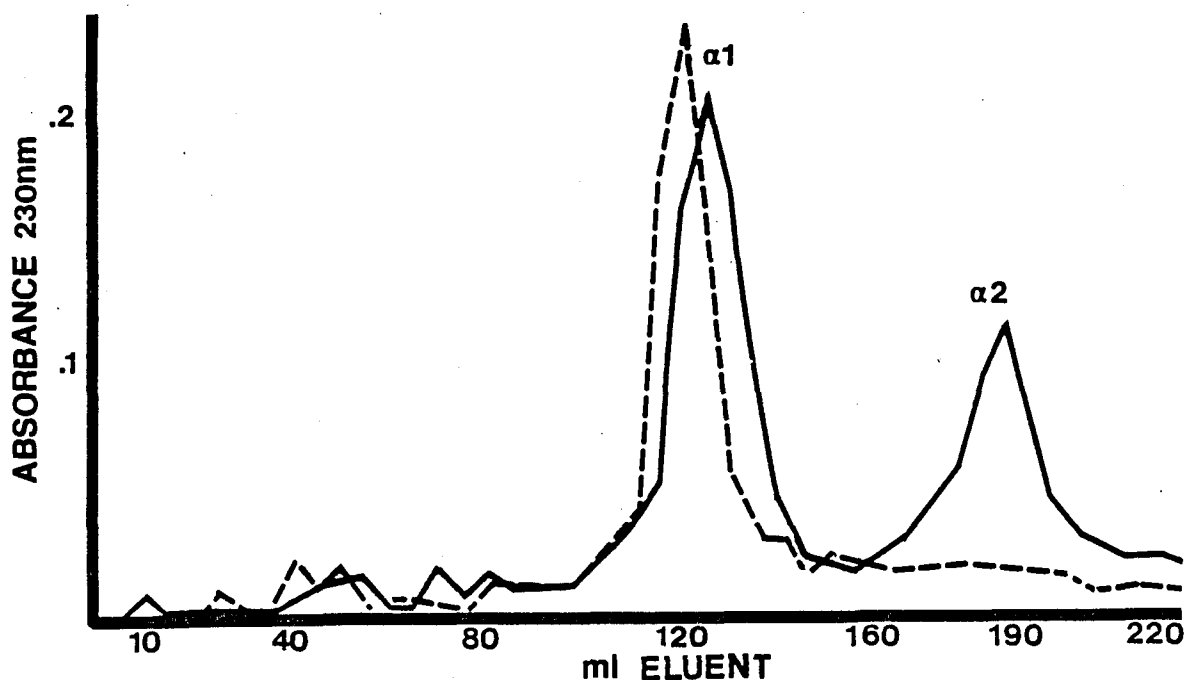


Figure 23. CM-cellulose chromatography of collagens separated by differential salt fractionation of tissues from the nonregenerating rabbit ear. Broken line—4.0 M extract of cartilage, solid line—2.4 M extract of skin and fibrous connective tissue.

Chromatography profiles of collagens extracted via differential salt precipitation of ear tissues at weekly intervals during regeneration are seen in the following three figures. The profile of the 2.5 M extract plus the 1.5 M extract (1.5 M precipitate was too small to risk chromatography alone) from the skin of a 14 day regenerate is seen in Figure 24. The skin at this stage, or wound epidermis, produces the expected Type I collagen with the $\alpha 1:\alpha 2$ ratio of 2:1. However note the additional small peak between $\alpha 1$ and $\alpha 2$. This peak may represent the small precipitate seen in the 1.5 M extract since columns without the 1.5 M extract added do not show this peak. To assure that this peak was not artifactual, an additional extraction of the 14 day regenerate skin was made using pepsin which will enhance the solubility of native molecules such as Type III and the same results were obtained. An attempt was made to characterize the small peak via SDS electrophoresis using the methods of Narayanan and Page (1976). When the 1.5 M precipitate was run on a SDS-polyacrylamide gel without mercaptoethanol the band stayed at the origin, with mercaptoethanol in the sample, the band migrated in the same position as the $\alpha 1$. Since Type III molecules have a disulfide bond within the helical portion of the molecule, they have a molecular weight of approximately 300,000 prior to reduction and 100,000 after reduction. The migration after reduction substantiates that this is a Type III collagen even though the band which migrated electrophoretically with $\alpha 1$ was so light that it could not be photographed.

The profile of the blastema region, precipitated at 4.0 M NaCl, from the 28 day regenerate is seen in Figure 25. The $\alpha 1:\alpha 2$ ratio is 5:1, indicating an over abundance of alpha chains unlike that seen in cartilage or skin. This profile is similar to the 4.0 M precipitate of the 35 day regenerate blastema.

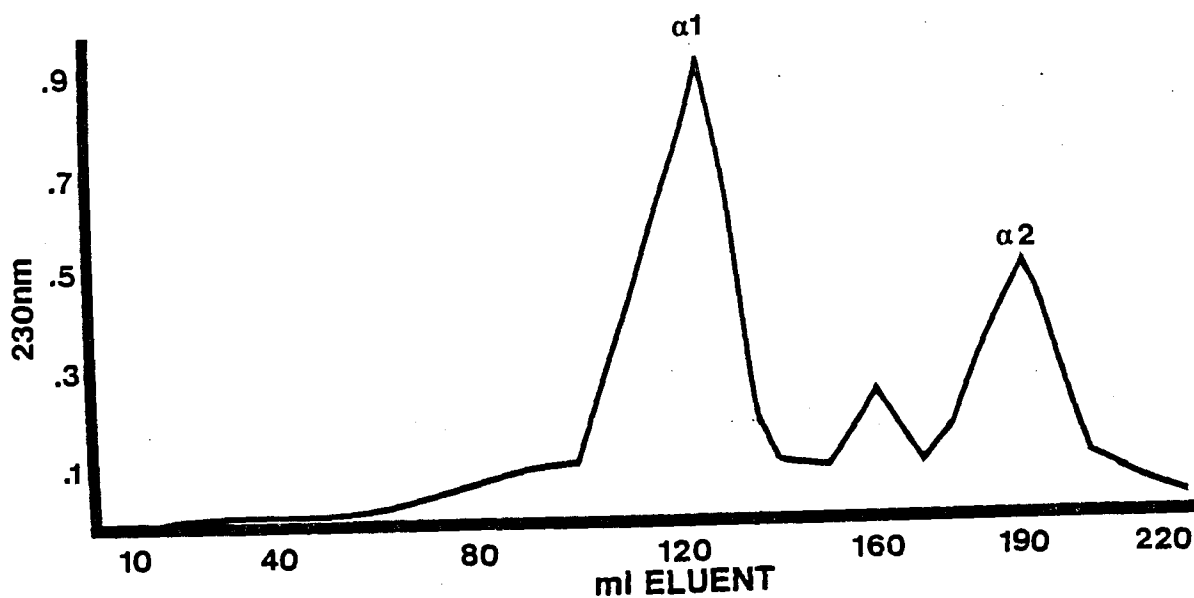


Figure 24. CM-cellulose chromatography of the collagen alpha chains of the 2.5 M and 1.5 M precipitates from differential salt fractionation of 14 day rabbit ear regenerating tissue.

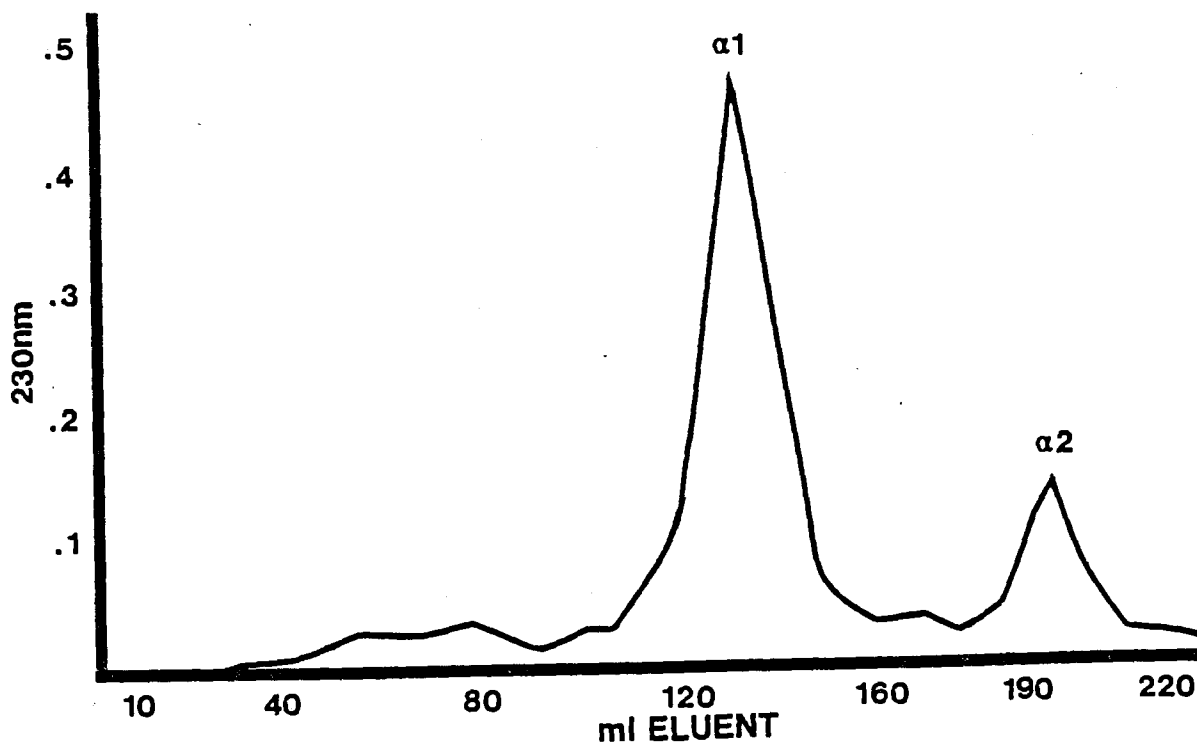


Figure 25. CM-cellulose chromatography of the collagen alpha chains of the 4.0 M NaCl precipitate from a 28 day rabbit ear tissue regenerate.

In Figure 26 one can see the profile of the 2.4 M precipitate of the skin from a 35 day regenerate with an $\alpha 1:\alpha 2$ ratio of 1.9:1, indicating Type I collagen. The profile of the collagen from the core blastema region i.e. where chondrogenesis is seen histologically, of a day 49 regenerate is seen in Figure 27. The sample was extracted at 4.0 M NaCl and has an $\alpha 1:\alpha 2$ ratio of 13:1, a pattern similar to Figure 23 of normal ear cartilage.

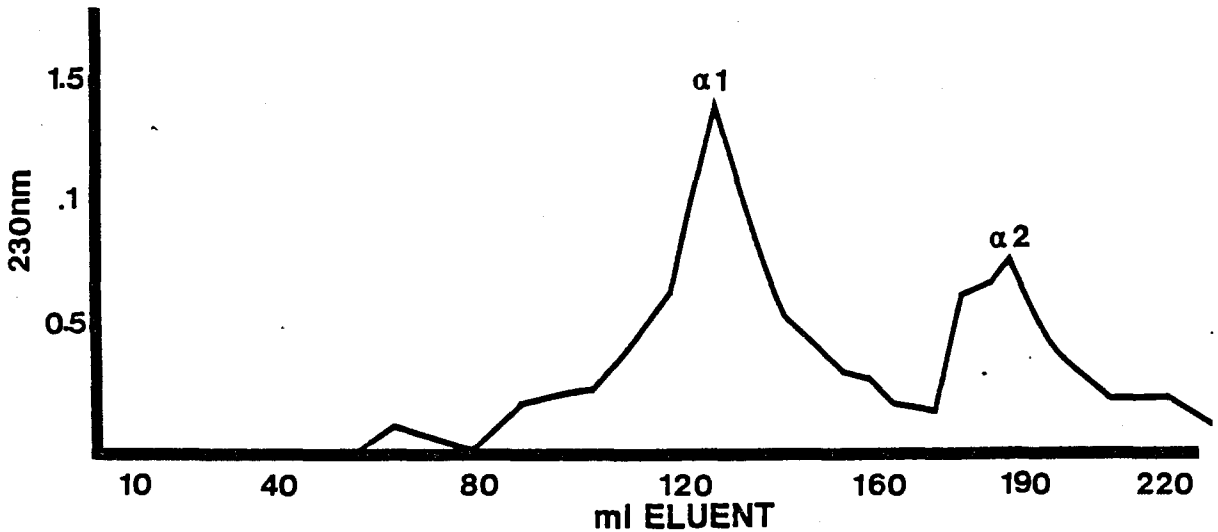


Figure 26. CM-cellulose chromatography of the 2.4 M NaCl precipitate of the skin from the regenerating ear tissues of a rabbit 35 days after punching.

Collagen typing: SDS-polyacrylamide gel electrophoresis. A number of the salt precipitates were analyzed by SDS-polyacrylamide gel electrophoresis to confirm the homogeneity of the peaks from the chromatography and from the salt extractions. Samples from different salt extracts and/or chromatography columns during the first 2 months of the regeneration process are seen in the following two gels.

In Figure 28, Lane 1 contains cartilage collagen (4.0 M precipitate) isolated from day 0 rabbit cartilage. Only one band appears which migrates in the same position as $\alpha 1$ of skin and connective tissue collagen which is seen

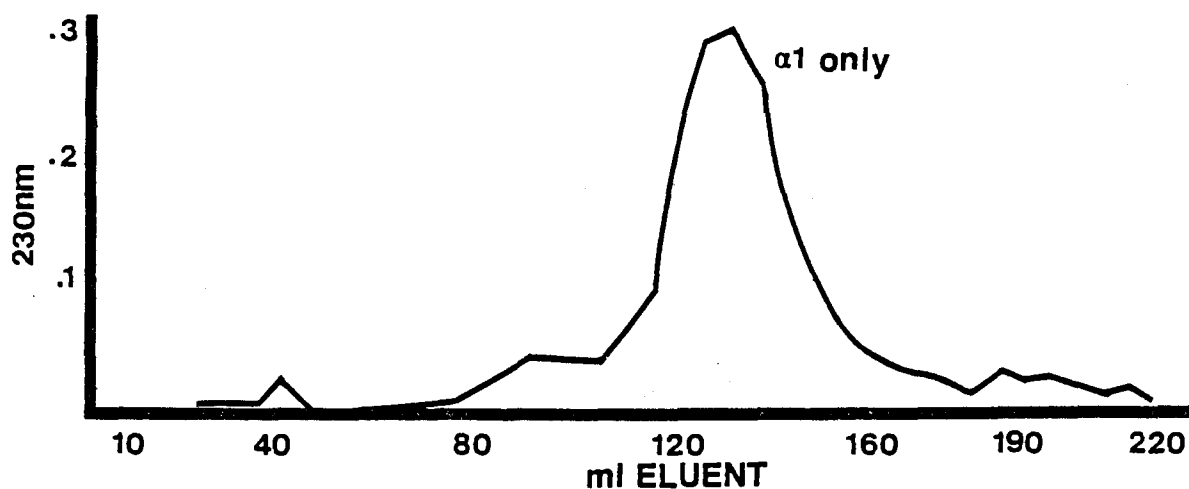


Figure 27. CM-cellulose chromatography of the collagen from the core blastema region of a 49 day rabbit ear tissue regenerate. The collagen sample is the 4.0 M NaCl precipitate of a differential salt fractionation.



Figure 28. Photograph of Coomassie blue stained alpha chains of collagens prepared by differential salt precipitation of regenerating rabbit ear tissues after fractionation by SDS-gel electrophoresis on 7.5% gels. Lane 1 = Type II collagen, 0 days; Lane 2, Type I collagen, 0 days; Lane 3 = Type I-Trimer, 35 days, Lane 4 = Type II 49 days after punching, $\alpha 1$ and $\alpha 2$ chains can be seen in lane 2.

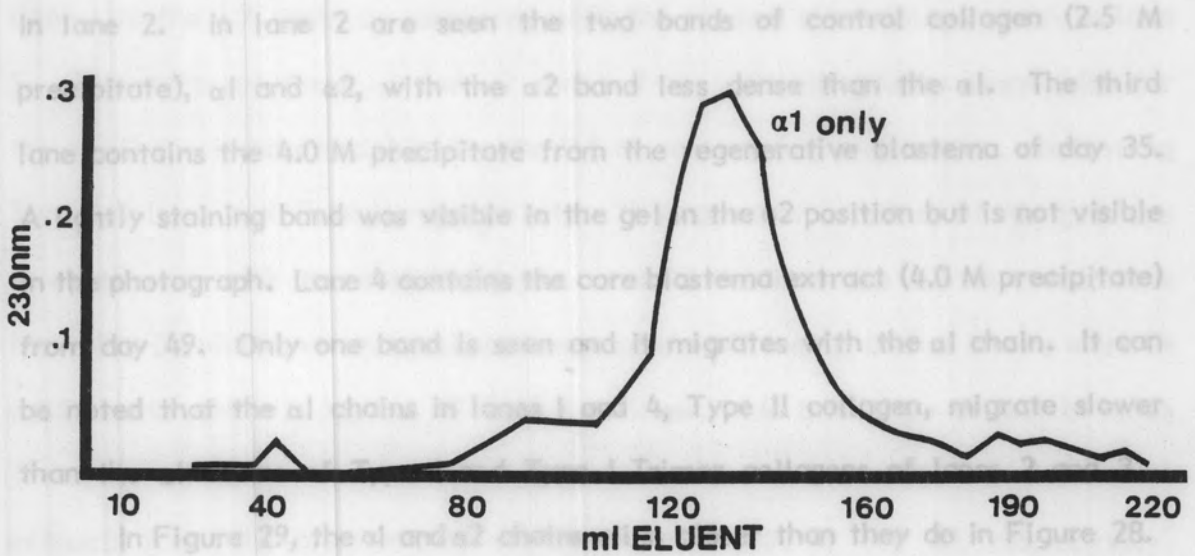


Figure 27. CM-cellulose chromatography of the collagen from the core blastema region of a 49 day rabbit ear tissue regenerate. The collagen sample is the 4.0 M NaCl precipitate of a differential salt fractionation.

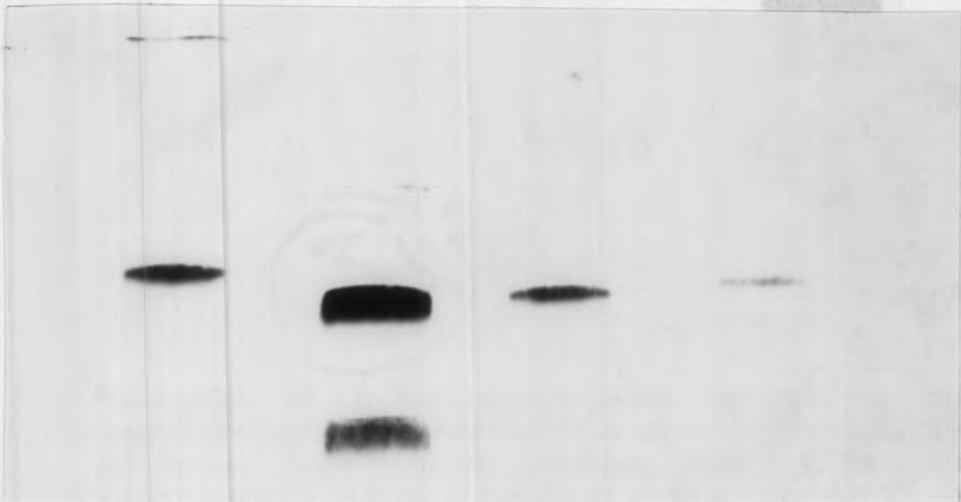


Figure 28. Photograph of Coomassie blue stained alpha chains of collagens prepared by differential salt precipitation of regenerating rabbit ear tissues after fractionation by SDS-gel electrophoresis on 7.5% gels. Lane 1 = Type II collagen, 0 days; Lane 2, Type I collagen, 0 days; Lane 3 = Type I-Trimer, 35 days, Lane 4 = Type II 49 days after punching, $\alpha 1$ and $\alpha 2$ chains can be seen in lane 2.

in lane 2. In lane 2 are seen the two bands of control collagen (2.5 M precipitate), $\alpha 1$ and $\alpha 2$, with the $\alpha 2$ band less dense than the $\alpha 1$. The third lane contains the 4.0 M precipitate from the regenerative blastema of day 35. A lightly staining band was visible in the gel in the $\alpha 2$ position but is not visible in the photograph. Lane 4 contains the core blastema extract (4.0 M precipitate) from day 49. Only one band is seen and it migrates with the $\alpha 1$ chain. It can be noted that the $\alpha 1$ chains in lanes 1 and 4, Type II collagen, migrate slower than the $\alpha 1$ chains of Type I and Type I-Trimer collagens of lanes 2 and 3.

In Figure 29, the $\alpha 1$ and $\alpha 2$ chains align closer than they do in Figure 28.

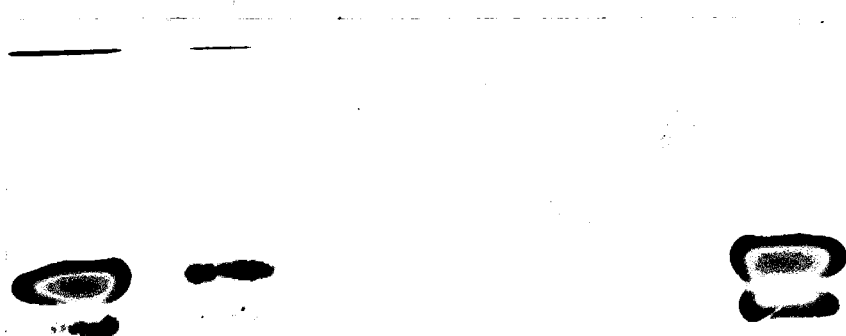


Figure 29. Photograph of a SDS-polyacrylamide gel of the collagen precipitated by differential NaCl in extracts of regenerating rabbit ear tissues. Lane 1, 28 day blastema; Lane 2, 35 day blastema, Lanes 3 and 4, 4.0 M precipitates of blastema day 14 and day 28; Lane 5, day 0 skin.

No explanation of this phenomenon is available. Lane 1 shows the 4.0 M precipitate from day 28 blastema whose chromatography profile is seen in Figure 26. Two bands are seen, the darker band corresponds to the $\alpha 1$ chain and the

in lane 2. In lane 2 are seen the two bands of control collagen (2.5 M precipitate), $\alpha 1$ and $\alpha 2$, with the $\alpha 2$ band less dense than the $\alpha 1$. The third lane contains the 4.0 M precipitate from the regenerative blastema of day 35. A lightly staining band was visible in the gel in the $\alpha 2$ position but is not visible in the photograph. Lane 4 contains the core blastema extract (4.0 M precipitate) from day 49. Only one band is seen and it migrates with the $\alpha 1$ chain. It can be noted that the $\alpha 1$ chains in lanes 1 and 4, Type II collagen, migrate slower than the $\alpha 1$ chains of Type I and Type I-Trimer collagens of lanes 2 and 3. In Figure 29, the $\alpha 1$ and $\alpha 2$ chains align closer than they do in Figure 28.

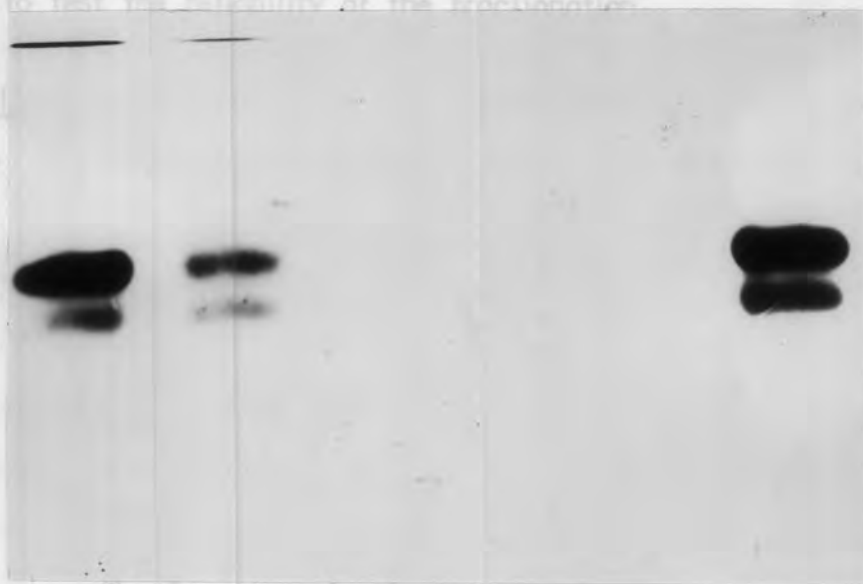


Figure 29. Photograph of a SDS-polyacrylamide gel of the collagen precipitated by differential NaCl in extracts of regenerating rabbit ear tissues. Lane 1, 28 day blastema; Lane 2, 35 day blastema, Lanes 3 and 4, 4.0 M precipitates of blastema day 14 and day 28; Lane 5, day 0 skin.

No explanation of this phenomenon is available. Lane 1 shows the 4.0 M precipitate from day 28 blastema whose chromatography profile is seen in Figure 26. Two bands are seen, the darker band corresponds to the $\alpha 1$ chain and the

lighter to the $\alpha 2$ chain as compared with the control skin collagen seen in lane 5. The ratios of these $\alpha 1$ and $\alpha 2$ chains from chromatography were 4.8:1 for the 4.0 M precipitate and 2.2:1 for the 2.5 M control skin preparation, confirming Type I-Trimer and Type I, respectively. The apparent densities of the gels are in agreement with the stated ratios. Lane 2 contains the 2.5 M precipitate from day 35 blastema whose chromatography profile of $\alpha 1$ and $\alpha 2$ chains were in a 1.9:1 ratio which is typical of Type I collagen. No bands are visible in lanes 3 and 4. This is not surprising since these are concentrates of the 4.0 M extracts from the blastema of day 14 and the skin of day 28, respectively. There were no precipitates in these extracts. These were used as internal standards to test the reliability of the fractionation.

Collagen levels. The total amounts of collagen found at each weekly time point until closure of the wound are seen in both Figure 30 and Table 4.

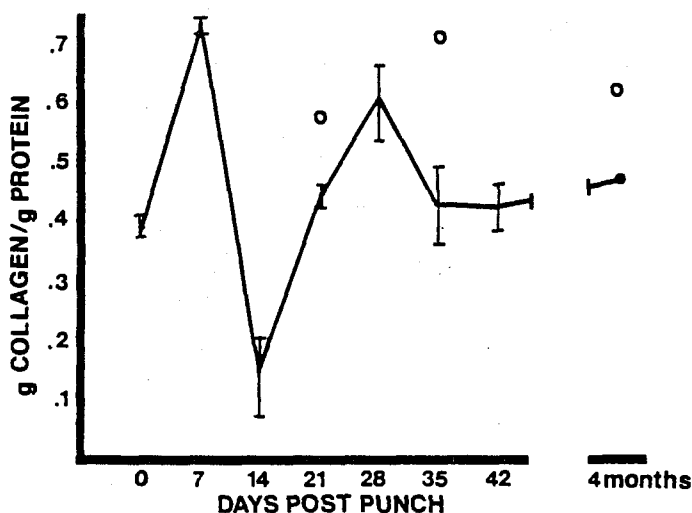


Figure 30. Plot of the g collagen/g protein as determined by hydroxyproline concentrations in acid hydrolysates of regenerating rabbit ear tissues at weekly intervals after punching. Open circles—data from animals that had stopped closing at those particular time intervals.

TABLE 4
COLLAGEN CONTENT OF EAR REGENERATION TISSUES*

Day After Punching	# Animals	# (ears)	$\frac{\text{g collagen}}{\text{g wet weight tissue}}$	$\frac{\text{g protein}}{\text{g wet weight tissue}}$	$\frac{\text{g collagen}}{\text{g protein}}$
0	5	(7)	.097 $\pm$.02	.242 $\pm$.04	.396 $\pm$.03
7	2	(4)	.142 $\pm$.01	.197 $\pm$.02	.735 $\pm$.02
14	4	(4)	.031 $\pm$.02	.203 $\pm$.04	.142 $\pm$.07
21	2	(2)	.049 $\pm$.01	.110 $\pm$.003	.450 $\pm$.02
NC	1	(1)	.077	.133	.549
28	4	(4)	.086 $\pm$.01	.143 $\pm$.02	.611 $\pm$.07
35	3	(5)	.082 $\pm$.01	.183 $\pm$.01	.435 $\pm$.07
NC	1	(1)	.112	.156	.718
42	2	(3)	.068 $\pm$.01	.159 $\pm$.01	.430 $\pm$.04
4 months	1	(1)	.137	.283	.484
NC	1	(1)	.171	.270	.633
Marginal 0 days	2	(2)	.057 $\pm$.01	.187 $\pm$.02	.318 $\pm$.03
Ear Punch 21 days	2	(2)	.094 $\pm$.02	.128 $\pm$.01	.764 $\pm$.04

*As determined by hydroxyproline analysis, Means $\pm$ S.E. mean
NC=punch not closing, i.e. remaining at near day 0 size.

The total amount of collagen found in the regenerating tissues varies during regeneration. In the normal ear tissue from day 0, approximately 40% of the total ear protein is collagen. By day 7 collagen is the predominant protein of the regenerating ear tissues. By day 14 the levels of collagen have decreased dramatically. The amounts of collagen are increased over the next two weeks to slightly higher than normal ear tissue levels. However between weeks 4 and 5 there is a loss of collagen to levels more similar to those of the nonregenerate. Note also that those animals who were not following the pattern of regrowth seen in Chapter II, i.e. who were not closing their wounds as rapidly as would be expected, consistently had greater amounts of collagen than those that were regenerating (open circles on graph).

One animal observed at four months post punching had one ear which closed and one which remained open. The closed ear contained the expected

amounts of collagen for a regenerated area which is equal to day 0 or slightly higher while the ear which had not closed had a higher level of collagen. Tissues sampled from marginal ear punches contained smaller quantities of collagen at day 0 which is not unusual since the edge of the ear has a much thinner cartilage layer than the more medial tissues. By day 21 the lost tissues had not been replaced and the wound remained open. These tissues contained large amounts of collagen, a factor which may contribute to their lack of regeneration.

Collagenase levels. Of the limited number of samples tested the pattern of collagenase activity correlates inversely with the collagen accumulation data, Table 5.

TABLE 5
COLLAGENOLYTIC ACTIVITY OF REGENERATING RABBIT EAR TISSUES

Days after Punching		Day of Culture			Number of Animals**
		1	2	3	
0		ND	ND	0.5*	3
7		ND	0.5	1.0	2
14		1.0	3.0	4.0	2
28		ND	0.5	2.0	1
35		0.5	1.0	3.0	2
Marginal Pinna Punch	0	ND	ND	1.0	2
	21	ND	ND	1.0	2

*Amount of lysis of collagen substrate, Scale = 1.0 - 4.0, 4.0 = complete lysis, ** 2 samples/animal (i.e. 1 per ear).

On day 0 the collagenase activity was scored 1 (in two of the three samples analyzed). The observation of collagenase activity in ear tissues after three days in vitro may simply indicate the initiation of inflammatory events.

By day 7 small amounts of lysis are detected but by day 14 the activity greatly increased and may be responsible for the decrease seen in total collagen at this time. Day 28 has a moderate amount of collagenase activity which may depict the balance between catabolism and anabolism. At day 35 another increase is seen in collagenase activity again correlating inversely with the decrease in collagen. Levels at 3 months are similar to control levels which may reflect the stabilization of the collagen matrix. Little collagenase activity is seen in wounds produced on the backs of rabbits or in marginal ear punch wounds. The low levels in the marginal ear punches may be responsible for the increased accumulation of collagen in these wounds.

Discussion

The results of the collagen study establish that significant biochemical changes occur in the regenerative blastema which do not occur in the tissues composing a simple wound, and that these changes are reflective of the state of differentiation in the tissues. These biochemical changes include the production of Type III collagen in the wound epidermal-dermal components of the regenerating tissues, a collagen type which is only seen in very small amounts in the skin of the adult organism, in tumors and is primarily seen in embryonic tissues. The finding of this collagen demonstrates that similarities exist between the regeneration blastema and other rapidly proliferating systems of growth such as tumorigenesis or embryogenesis.

The other unusual type of collagen, Type I-Trimer, is only seen when metabolic changes are occurring in the collagen matrix. Its discovery in the regeneration blastema is exciting since it indicates that the pre-cartilage cells are following similar developmental transitions which occur in the embryo or during any rapid turnover of collagen, but not repair of tissues with simple

scarring. Our discovery of Type I-Trimer in the ear substantiates the work done in vitro by Smith and Kang (1981) and demonstrates that the presence of this collagen in the regeneration system is not simply a change seen in an in vitro synthesis system. These changes in collagen types with regeneration, from Type II in cartilage to Type I and Type I-Trimer and back to Type II, lead one to suggest that the cartilage or associated cells have returned to a more embryonic cell type.

Secondly, the changes seen in the levels of collagen deposited in the regenerating tissues are different from those of marginal ear wounds or back skin wounds. These differences may reflect the capacity of the former to regenerate and the latter to form a scar. Initially the deposition of a large quantity of collagen is necessary to rapidly cover the wound and restore the strength and rigidity to the damaged tissues. The second accumulation of collagen may place a morphological constraint on cell movements if oriented against the direction of cell movement and prevent differentiation into the original cell types and patterns. As suggested in the last chapter perichondrial proliferation may provide the mass of cells that will reestablish the cartilage layer in the ear. If the perichondrial cells are blocked by the mass of collagenous fibers they cannot proliferate and reform the lost cartilage. The second accumulation is removed by around day 35. After 1 month tissues differentiate histologically, particularly cartilage.

The collagenase pattern relates inversely to the changes seen in collagen levels, i.e. being low when collagen is high, low at the beginning of the process and high as collagen decreases. This rapid proliferation of collagen may help fill the lesion as rapidly as possible, as occurs in most wounds, but unlike most wounds, there is not the sustained increase in collagenase accompanying this event, although there is an initial increase to remove the wound debris.

However the levels of collagenase do not increase again after the cicatrix (scar) is deposited and the resulting tissues which fill the wound are not like the original tissues.

Type I collagen is normally found in the skin and connective tissue, and Type II is found in the cartilage of the rabbit ear. With regeneration the skin continues to produce Type I and at certain times small amounts of Type III. The blastema produces Type I and Type I-Trimer early in regeneration but with the differentiation of histologically visible cartilage, Type II is produced once again. Neither Type III nor Type I-Trimer is found in dermal wounds or tissues replaced after a marginal pinna punch. These changes seen in the type of collagen produced by the regenerating ear tissues are unusual in adult tissue repair but are not unusual in development.

Transitions in Collagen Type with Development

In the present study the type of collagen produced by the regenerating ear tissues was used as a sign of cartilage differentiation. Transitional changes in embryonic collagen are considered indicative of the tissues' state of differentiation (Miller et al., 1971; Trelstad et al., 1973; Linsenmayer et al., 1972a, 1973c). Supportive evidence for the use of collagen as an indicator of differentiation can also be found in repair processes. The repair of surgically induced defects in mature rabbit hyaline cartilage was seen to originate from the subchondral bone (Cheung et al., 1980) and this repaired cartilage synthesized Type II collagen. Since the repaired tissue originated from the subchondrial connective tissues, such as osteoblasts, mesenchymal cells, and fibroblasts which produce only Type I collagen, a change in the biochemical phenotype of one or more of these cell types had to occur to get Type II. Thus the changes in collagen type were reflective of the state of cartilage differentiation.

In the immunofluorescence study of collagen synthesis during development of the long bones of the chick embryo, von der Mark and colleagues (1976a, 1976b) demonstrated that cells switch from Type I to Type II collagen synthesis during three separate developmental processes and Reddi et al., (1977) reported a similar transition in collagen phenotype during matrix-induced endochondrial bone morphogenesis.

We suggest that the regeneration of the rabbit ear cartilage follows a similar sequence of changes and that these changes are indicative of the state of differentiation in the tissues. If the cartilage arises from proliferation of chondrocytes Type II collagen would be seen throughout regeneration and no alteration in differentiation would exist. If the cells which give rise to cartilage are dedifferentiated from chondrocytes and pass through mesenchymal- or fibroblastic-like stages, Type I would be seen. Not only is there a transition from Type II to Type I but the Type I-Trimer, which is suggested to be in the normal development sequence of collagen, is also seen. However, since perichondrium produces Type I the new chondrocytes may very likely be perichondrial progeny. If this explanation is true one would also expect to see a transition from Type II of the original cartilage to Type I of the expanding perichondrium and back to Type II in the differentiation of the perichondrium to cartilage. This transition is seen with the addition of Type I-Trimer. Type I-Trimer is not normally seen in the appositional or embryonic growth of cartilage. This evidence suggests that if the cartilage is regenerated from perichondrium it does not occur in exactly the same manner as it does in the normal appositional growth of cartilage or during embryonic cartilage development.

Type I-Trimer

The extraction of Type I-Trimer from the blastema tissues demonstrates

that the blastema is different biochemically from normal dermal wounds and its presence may be an indication of an altered phenotype of its producing cells. Type I-Trimer synthesis has been demonstrated by cultured cells under conditions which allowed the cells to 'dedifferentiate' to their mesenchymal precursor form (Benya et al., 1977) and cultures of chondrocytes given embryo extract or maintained for an extensive time in culture produced Trimer (Uitto, 1979). Under such conditions the cells which initially synthesized only Type II collagen, switched their collagen production to predominately Type I and Type I-Trimer. Most of the reported instances of Type I-Trimer synthesis are by cells in culture and cells with presumably altered metabolic states. However, the Type I-Trimer cannot be simply a product of cells established in tissue culture, since normal cultures of fibroblasts do not synthesize significant amounts of the Type I-Trimer (Narayanan and Page, 1976). Conversely, cultures of freshly isolated normal rat incisor odontoblasts do synthesize Type I-Trimer (Munksgaard et al., 1978). Type I-Trimer has also been isolated from EDTA-demineralized experimentally produced lathvritic rat incisors (Wohlleb and Carmichael, 1978), and in normal human skin, amounting to less than 5% of the total collagen (Uitto, 1979).

The significance of Type I-Trimer synthesis in adult tissues and tumors is not known. Maynes et al., (1975, 1976) suggest that the synthesis of Type I-Trimer results from altered gene expression due to perturbations of the transcriptional process by agents such as bromodeoxyuridine, factors in embryo extract, or in association with in vitro cellular senescence. Since we found Trimer in the tissues of adult animals there apparently occurred some alteration in the normal production pathway of collagen thereby distinguishing the regeneration event from wound healing and making the process more similar to embryonic development.

The present study conducted in vivo substantiates the in vitro work of Smith and Kang (1981) who isolated Type I-Trimer from cultures of the regeneration blastema of the rabbit ear. Trimer was not found in our studies in healing wounds, healing tissues of punches placed on the margins of rabbits ears, or in normal ear cartilage. Finding Type II cartilage in nonregenerated or normal ear cartilage and in newly regenerated cartilage supports the suggestion that Trimer synthesis is within the normal pathway of embryonic cartilage development.

Type III Collagen

The presence of Type III collagen in the regenerating tissues may demonstrate that similarities exist between embryogenesis and regeneration since Type III is considered an embryonic collagen. Type III collagen was not found in dermal back wound tissues nor in the tissues of a nonregenerating marginal ear punch-wound. Similar results were seen in a comparison of guinea pig insoluble normal and scar collagen: Type III was found in small quantities in normal skin but none was found in dermal scar tissues (Shuttleworth et al., 1975). The presence of Type III collagen in embryonic skin has led to the conclusion that this collagen is laid down in the embryo and gradually replaced by Type I during development. However since fibroblasts can make both Types I and III collagen (Moro and Smith, 1977) the production of this collagen in the adult means that the fibroblasts have reverted to the synthesis of a more embryonic type collagen and are showing a dedifferentiated phenotype.

Collagen Levels and Collagenase

With the present study a relationship between collagen levels and the activity of a collagenolytic enzyme is seen. Similar interactions between a matrix component and its specific enzyme have been observed in newt limb

regeneration. Toole and Gross (1971) report the time course of synthesis of hyaluronate in the early blastema and the removal of this polysaccharide by the action of an endogenous tissue hyaluronidase at the onset of differentiation of precartilage. Grimes (1981) observed the same interactions on the role of hyaluronate and hyaluronidase in cell migration during rabbit ear regeneration; hyaluronate was present in the path traversed by the migratory cells and a marked increase in hyaluronidase activity was seen to coincide with both the cessation of this migration and the onset of chondro- and fibro-genesis. Chondrogenesis commences in the fourth week in the regenerating rabbit ear tissue (Grimes, 1981) and it is between weeks 4 and 5 that we see both a decrease in total collagen as well as the second increase in collagenase activity. Similar changes in collagenolytic activity were found in the regenerating newt forelimb tissues at the time of differentiation (Grillo et al., 1968). The removal of a portion of the matrix collagen through the action of collagenase may be necessary for differentiation to commence.

What regulates collagenase production during regeneration is not known but the interaction of the epithelium and the underlying mesodermal tissues is suspected. Johnson-Muller and Gross (1978) suggest that direct cell contact or close proximity allow the two cell types to interact in collagenase production and that in the cornea the epithelial cell stimulates the stromal cell to produce the enzyme. Epithelial-mesenchymal interactions are fundamental to morphogenesis in embryonic development (Slavkin et al., 1977), limb regeneration (Carlson, 1974), and wound repair (Peacock and Van Winkle, 1976). Control mechanisms for collagen degradation that depend on interactions between tissue cells, as in wound repair, may involve a finely balanced mixture of stimulatory and inhibitory factors. Recently, Johnson-Wint (1980) found that media conditioned by epithelial cells from the adult rabbit cornea were capable of

both stimulating and inhibiting production of latent collagenase by stromal cells of the cornea. Thus epithelial cells may be capable of modulating collagen degradation in the cornea, and perhaps in all integument, a regulation which may be essential to wound healing and regeneration. Grillo and Gross (1967) demonstrated high levels of collagenolytic activity in cultures, wounds in guinea pigs and, also from proliferating marginal epithelium alone but not from dermis or unwounded connective tissue. However granulation tissue from the center of an open wound was much less active, perhaps due to the lack of interaction with the epithelium.

These results agree well with our findings of collagenase activity in the skin wound of the rabbit at 14 days. The skin wound did not produce the large amount of activity seen in the 14 day blastema. As exhibited by those animals thought not to be regenerating the amounts of salt extractable collagen remained higher than those were closing. These higher levels may reflect a lower quantity of collagenase or a higher collagen synthesis rate. One can speculate that the second increase in collagenolytic activity seen on day 35 will not occur in those animals where the ear is not regenerating and that the excessive accumulation of collagen within the granulation tissue may be due to the lack of a continued production of collagenase.

As well as being controlled by its interactions with other tissues, collagenase may also be hormonally controlled. Progesterone has been shown to inhibit the normal release of collagenase into the media of cultures of post-partem rat uterus (Jeffrey et al., 1971). Neither estradiol nor testosterone diminished the collagenase activity in vitro although estradiol enhanced progesterone's inhibition. These data on the hormonal control of collagenase production may be valuable in explaining results seen in our initial studies, in Chapter II. The lower success rate of ear regeneration seen in females may

reflect the inhibition of collagenase allowing high levels of collagen to be retained which may prevent the outgrowth and differentiation necessary for regeneration.

Our initial collagen accumulation data is what one would expect in wound healing. As shown in the healing wounds of the guinea pig in the carrageenan granuloma and in implanted sponges (Gross, 1958) it takes 5-7 days for significant amounts of collagen to appear. The fluctuations in collagen levels are similar to those seen in the regenerating limbs of adult newts (Schmidt, 1970) and may be attributed to the changes in the levels of the collagenolytic substance found in the cultures (Grillo et al., 1968). Collagenase activity is not seen in unwounded tissues. Therefore, the observation of lytic activity in all the cultures is puzzling since the day 0 tissues were not from wounds. However under culture conditions, the tissues are viable and should respond to the damages at the edges where the punches (wounds) were made. Since collagenase enzymes are not known to be stored internally (Johnson-Wint, 1980), the delay before the slight response may reflect the initiation of normal wound healing processes.

Role of Collagen

The transitions in types of collagen produced, the amounts produced, and the activity of the collagenolytic enzyme separate rabbit ear tissue regeneration from simple wound healing. Collagen may serve as a matrix component which directs development or it may simply be the result of a developmental change. The exact role of collagen in this system is not known but its role may be similar to that seen in other tissues. Collagen and glycosaminoglycan produced by embryonic tissues are useful markers in development and may be active agents in tissue interactions leading to

morphogenesis. In the developing chick eye accumulations of vitreous materials, such as collagen and glycosaminoglycans, provide the expansive pressure for the development of normal eye geometry (Coulombre, 1965; Newsome et al., 1976), and the synthesis of sulfated glycosaminoglycans by the embryonic corneal epithelium is stimulated by several collagen types (Meier and Hay, 1974). Collagen has been shown to direct development in cell cultures: the growth of fibroblasts is inhibited in vitro by preventing collagen secretion by these cells (Liotto et al., 1978); the ultrastructural and biochemical differentiation of mammary epithelial cells in culture is influenced by floating collagen substrates (with the appropriate hormones) (Emerman et al., 1979); and capillary endothelial cell proliferation by a tumor angiogenesis factor is stimulated by a collagen substratum (Schor et al., 1979).

Lash and Vasan (1978) found that the most effective inducer of somite chondrogenesis in vitro was Type II collagen, the same type of collagen synthesized by the in vivo inducer, the notochord. It has been suggested that these matrix components exert their apparent regulatory effects by interacting with components of the somite cell surface. In fact, the role of the notochord in somite differentiation is to produce collagen which lowers the cAMP levels (an increase in cAMP prevents somite differentiation) in the somites and thereby triggers chondrocyte differentiation (Kosher and Savage, 1979). Hay (1973) suggested that different kinds of collagen enable embryonic tissues to control the shape and differentiation of adjacent tissues and that in turn the matrix interacts with cell membranes to stabilize cell differentiation. Removal of these investing layers leads to faulty differentiation. We can suggest that in the rabbit ear regeneration system collagen may participate in the direction of differentiation and may also serve as a scaffolding for directing cell migration (Fig. 18, page 53).

Collagen may induce cells to manufacture their extracellular material and specific cell proteins which control the cellular shape. Corneal epithelial cells grown on plastic have a fibroblastic, flattened configuration and will not respond to epithelial growth factor but will respond to fibroblastic growth factor: When grown on collagen substrates they become tall and columnar and respond primarily to epidermal growth factor (Gospodarowicz et al., 1978). Since rabbit dermal wound tissues do not produce the types of collagen seen in the regenerating ear tissues, it may be that only certain collagen types are able to influence growth and differentiation and these changes may reflect the capacity of the ear tissues to regenerate while other tissues cannot.

The metabolism of collagen in the regenerating rabbit ear tissues was studied and significant contributions were made in establishing the process as unique. Two types of collagen were found which are not found in the regenerating tissues of other animals but are found in other developmental systems. Transitional collagen changes may signify that biochemical dedifferentiation of the cells composing the regenerate occurs. Additionally, changes were seen in the amounts of collagen produced and evidence for a collagenolytic enzyme was observed. The interactions of these two molecules may be critical to the success of regeneration since in ear tissues that did not regenerate increased amounts of collagen and decreased amounts of collagenase were found. This accumulation of collagen may block regeneration. From the biochemical typing information it can be suggested that the regeneration system is more similar to embryonic, tumorigenic, and tissue culture cell systems than to wound healing. This evidence supports our original hypothesis.

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ANALOGIES WITH EMBRYOGENESIS

Introduction

Although regeneration is a developmental process, this process is occurring in an environment of highly differentiated tissues. Yet the mechanisms of regeneration are more similar to those seen in embryonic growth than in the growth for repair and maintenance seen in the adult. A review of regeneration reveals analogies with developmental processes; the differentiating tissues of the regenerating amphibian appendage for example appear to rely on many of the molecular activities found in developing organs (Schmidt, 1968). As stated previously, the origin of the blastema cells and the tissues that form a new limb reside within the amputation stump (Butler, 1933; Chalkley, 1956). These origins are in no way similar to the origins of embryonic cells yet developmental processes are featured in both repair and regeneration in vertebrates. Our original aim in looking at similarities between the regenerating ear tissues and the environment of the preimplantation rabbit uterus, was to find an extrauterine site in which to grow rabbit embryos. Since regeneration is another system of rapid growth and differentiation in mammals comparisons between this system, embryogenesis and tumorigenesis could be made in hopes of fulfilling this aim. The present study investigates those changes in the milieu of the embryo that might also be present in regenerating tissues. The factors present in the uterus that seem to relate to growth and development of the embryo may also be present in the ear to stimulate the development of the embryonic-like blastema cells. Two such factors were investigated, namely; uterine proteins and gallium binding to an undetermined factor.

Secretory uterine proteins have been described which vary with the stage of the cycle, or stages of pregnancy, being particularly prevalent during the time when the embryo is undergoing its initial differentiation. It is suggested that these proteins may serve a major role in early development. When the protein composition of the uterine fluid was altered by progesterone injection into rabbits two days before mating and subsequent normal fertilization, the resultant embryos died by day 4. McCarthy et al. (1977) concluded that the asynchrony of the uterine fluids was embryotoxic.

The most widely studied uterine protein is uteroglobin (blastokinin). It is a major secretory protein found mainly in the uterus during the preimplantation stages of embryonic development of the rabbit (Krishnan and Daniel, 1967; Urzua et al., 1970; Beier, 1968). There is controversy over whether uteroglobin (UTG) supports blastocyst growth in vitro or not (El Banna and Daniel, 1972; Maurer and Beier, 1976) but it binds progesterone which may affect blastocyst development (Arthur and Daniel, 1972; Beato and Beier, 1975). Recently a hypothesis for its role in embryogenesis has been developed which suggests that uteroglobin is an anti-immune factor, in conjunction with other uterine components, serving to prevent the mother from rejecting the embryo (Mukherjee et al., 1980).

Regeneration and tumorigenesis have certain elements in common; therefore the environment needed for regeneration may be expected to be similar to that required for tumorigenesis. Prehn (1971) suggested that tumors may be futile attempts to regenerate and thus these two systems may share common characteristics and growth requirements. It has been observed that tumors are easier to induce on the body of a newt than on the tail which can regenerate; a virus induced kidney carcinoma of Rana pipiens may revert to a normal differentiation when grown in the regenerating region of tadpole

tails; and the proliferation of very fine nerves into the epithelium after treatment with oncogenic hydrocarbons is similar to the development of the blastema which is also preceded by the proliferation of fine nerves into the epithelium of the apical cap of the regenerating limb (Mattson, 1976).

Gallium-67, a radionuclide used clinically to localize tumors, has been shown to bind to placental tissues in primates (Fogh, 1971), mice (Otten et al., 1973) and in rabbits (Tyndall et al., 1976). The label is also associated with lactating breast tissues (Fogh, 1971) preimplantation rabbit blastocysts and the macromolecular fraction from Sephadex G-200 chromatography of uterine flushings collected on the 5th day post coitum (Tyndall et al., 1976; Dunbar and Daniel, 1979). Gallium has also been localized in normal rat liver and in Morris 5123C hepatoma homogenates (Brown et al., 1976).

In view of the fact that Gallium-67 localizes in both tumor tissue and embryonic tissue or those tissues which will support the embryo, and that similarities exist between tumor tissues and regenerating tissues, we hypothesized that Gallium-67 would also bind to the undifferentiated tissues of the regeneration blastema.

Materials and Methods

Tissue collection. Tissues were collected by ear biopsy at weekly intervals from the day of punching through day 72 by methods described in Chapter II, p. 21 and 22. The tissues were placed in PBS at 4° C, minced, homogenized with a motor-driven teflon pestle on ice, centrifuged for 15 minutes at 2000 x g and the supernatant removed and used immediately or frozen for later analysis. Total soluble protein was analyzed by the method of Lowry et al. (1951).

Electrophoresis. Samples from the above supernatants were dialyzed against running buffer and subjected to discontinuous step gradient (8, 6, 4½ %) polyacrylamide gel electrophoresis using 0.375 M Tris sulfate separating gel buffer and 0.065 M Tris borate running buffer. The gels were run at 325 volts with a pulsed power source. Following electrophoresis the gel slabs were fixed and stained in 0.1% amido black in 10% acetic acid for 4 hours and then destained in 10% acetic acid until cleared, and photographed.

Immunological analysis (IA): collection of uterine proteins. Five mature virgin, New Zealand does were bred to 3 males each. On day 5 post coitum the animals were killed by cervical dislocation or air embolism, their abdomens opened and the uterus exposed. The utero-cervical junction was cut and cannulated. Each horn was flushed with 5 cc cold phosphate buffered saline (PBS) injected at the utero-tubal junction and collected from the cannula into conical centrifuge tubes. The PBS flush was followed by 5 cc air to remove any remaining fluid. The flushings were checked for blastocysts and the ovaries checked for corpora lutea to guarantee the stage of early pregnancy. The clear fluid was removed leaving the cell debris and blastocysts at the bottom of the tube. The fluid was centrifuged at 4800 x g at 4° C for 20 minutes. The supernatant was removed, placed in Spectrapor 3 membrane, dialyzed against 2 changes of cold distilled water for 24 hours, and concentrated in Aquacide (Calbiochem) to a volume of 1 ml. An aliquot was removed and the protein determined.

IA: chromatography. The concentrated sample was chromatographed on a column of Sephadex G-200, 2.5 cm x 38 cm, as described by Arthur and Daniel (1972), and eluted with 0.01 M phosphate buffered saline (0.9% NaCl, pH 7.2) with 0.01% sodium azide as an antibacterial agent. 1.9 ml fractions

determined as listed above. The profile of the column is seen in Appendix II. Fractions were also read spectrophotometrically at 280 nm. The samples were pooled into high molecular weight, albumen, and uteroglobin fractions, concentrated in Aquacide at 4° C, and stored frozen.

The uteroglobin fraction was rechromatographed on a 1.5 x 30 cm column of Sephadex G-50, 0.75 ml fractions were collected, and the protein concentration determined as above (Appendix III). The portion of the center of the peak was fractionated electrophoretically as described previously. After electrophoresis the last lane of the gel slab was sliced off the remainder of the gel and stained in amido black for 20 minutes until the prominent uteroglobin band was visible. The remainder of the gel was retained on ice. The stained slice was laid in its original position relevant to the rest of the lanes so the location of the uteroglobin could be identified. The region of the uteroglobin band in all the lanes was removed to be used for antibody production then the remainder of the gel was stained to confirm that only the band where the uteroglobin migrates was removed (Appendix IV). This same isolation procedure was performed for the amino acid analysis of UTG and the sample contained only one species of protein (Popp et al., 1978).

IA: immunization and antibody recovery. The uteroglobin portion of the gel was homogenized and aliquots were removed containing 100 µg of protein, emulsified with 0.5 ml of Complete Freund's Adjuvant (Difco), and injected into multiple intradermal sites and subscapularly into mature guinea pigs. Two weeks after the initial injection, the guinea pigs received a subcutaneous booster of 100 µg of the gel uteroglobin emulsified with 0.5 ml Incomplete Freund's Adjuvant. Two weeks after the booster, 5 cc of blood was collected by cardiac puncture and 2 weeks later the animals were

exsanguinated by cardiac puncture. The blood was allowed to clot overnight at 4° C, and the serum removed, spun at 2000 x g and stored frozen. The antisera was absorbed against rabbit serum, and liver and lung homogenates overnight at 4° C. The serum was removed by centrifugation at 6000 x g. The anti-high molecular weight protein antibody was produced in our laboratory using similar methods including absorption to make it uterine specific (Dunbar and Daniel, 1979).

Immunodiffusion. Immunodiffusion was performed according to the method of Ouchterlony (1958, as cited in Garvey et al., 1977). 2.5 ml of a solution of 0.8% Agarose (Sigma) in 0.005 M phosphate buffered saline pH 7.45 were pipetted onto glass slides and allowed to harden. Six wells 4 mm in diameter including a center well were cut into the agar. Samples of the soluble proteins from the ear homogenates were placed in the outside wells and the antiserum was placed in the center well. The plates were placed in a humidified chamber with sodium azide and allowed to develop at room temperature for 24 hours. After running, the wells were rinsed with distilled water and soaked for 5 hours in 0.3 M NaCl, changing it several times and soaked overnight in 0.15 M NaCl to remove unreacted proteins. The slides were then dried to a non-hydrated film, stained for 15 minutes in Crowles Triple Stain, destained in 2% acetic acid and preserved in 1% glycerin. The slides were then photographed. Other slides, not stained, were photographed immediately after developing using dark field illumination.

Immuno-electrophoresis. Immuno-electrophoresis was performed by a modification (Pan, M. L., personal communication) of the method of Graber and Williams (1953). Ten ml of 0.8% agarose in barbital buffer (sodium barbital 15.85 g, 0.1 N HCl-230 ml and DH₂O-770 ml) pH 8.2, were poured onto 2x2

inch slides, allowed to set for 15 minutes at room temperature and 30 minutes in a humid chamber. Samples were placed in cut wells and separated by electrophoresis at 240 volts for 1 hour. A center trough was cut from the slide and the space filled with antisera. The slides were allowed to develop for 24 hours at room temperature. They were then stained by the same method as the immunodiffusion slides above.

Rocket immunoelectrophoresis. A solution of 1% Agarose in 0.1 M Tris-sulfate buffer was prepared and retained in a water bath at 55° C. A confluent layer was then poured between two subbed microscope slides separated by spacers. The gels were allowed to set for 5 minutes. Wells were cut in the agar on the short side of the slides, filled with either uteroglobin standards or soluble proteins from the ear homogenates, and allowed to diffuse for 45 minutes. To the warm agarose solution was added 80 μ l of uteroglobin antibody per 2 ml of agarose solution. A section of the plain agarose was removed just distal to the sample wells, covered with another slide, and the space between the slides filled with the agarose-antibody solution. This solution was allowed to polymerize for 5 minutes and then subjected to electrophoresis at 100 volts for 3 hours in a 4° C refrigerator. The gels were placed in PBS overnight to remove unreacted protein, then dried by covering with filter paper and placing in an incubator for about 2 hours. The gels were stained in Coomassie Blue for 15 minutes.

Gel filtration. Soluble proteins from the regenerating ear tissue homogenates were fractionated on 1.5 x 30 cm Sephadex-G-200 columns by the same methods as described for the larger columns.

Gallium localization. As a preliminary study, 1 mCi Gallium-67 was injected intravenously into one rabbit with its ears bearing punches of two

ages. One punch was completely regenerated, the other was on day 20 post punch. The following day tissue was removed, the various regions homogenized, including non-punched sections, regenerating tissues and sections that had already regenerated, and counted with a gamma counter.

Results

Since Gallium-67 localizes in tumors and embryonic tissues, or those tissues which will support the embryo, the binding of Gallium-67 to regenerating tissues was tested. The blastema was found to incorporate five times as much Gallium as non-blastema regions. Both nonpunched regions and regions that were completely regenerated showed little Gallium-67 localization (Figure 31).

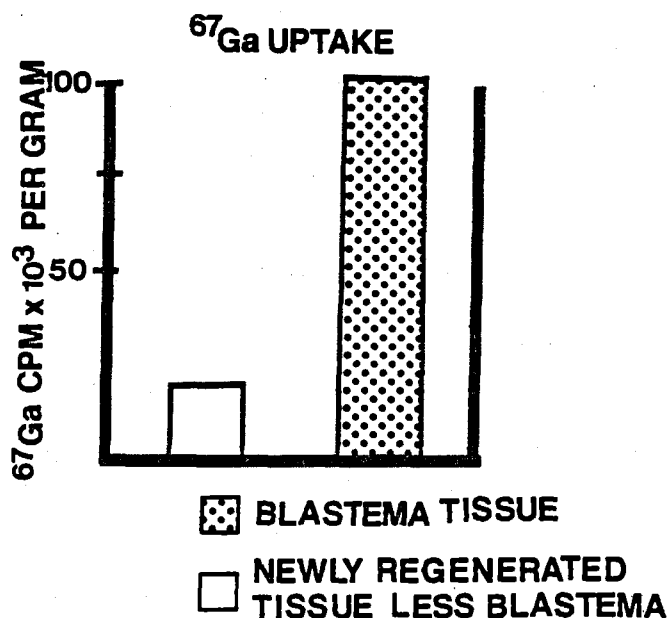


Figure 31. Localization of Gallium-67 in the regenerating ear tissues of a single rabbit. Unmarked bar represents that amount of the isotope found in a tissue section that had just completed regeneration. Stippled bar represents blastema tissue 20 days post punch.

With the information that Gallium-67, which binds to a high molecular weight protein from uterine flushings of the preimplantation stages of pregnancy

(Dunbar and Daniel, 1979), also localizes in the regenerating tissues, the soluble protein extracts from each week of the regeneration process were tested for the presence of antigens similar to those seen in the uterus during the preimplantation stages, in particular the protein uteroglobin. When the ear proteins were subjected to immunodiffusion against the anti-uteroglobin antibody, the samples from days 14, 28, 35 and 42 showed precipitin bands against the antibody (Figure 32). Day 0 and dermal wound tissues showed no bands. The faint bands can be seen at the tips of the arrows and do not show identity with the uteroglobin standard in well 6.

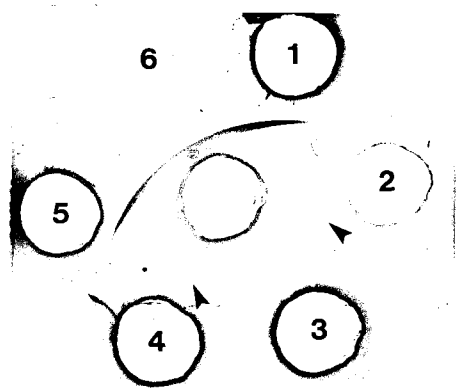
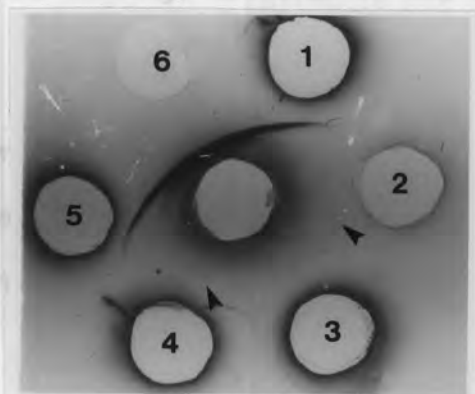


Figure 32. Ouchterlony double immunodiffusion. Soluble proteins from extracts of regenerating ear tissues run against uteroglobin antibody. Well six contains uteroglobin standard, Wells 1-5 contain, 0 day punch, 14 day regenerate, 28 day, 35 day, and 42 day regenerating ear tissue extracts, respectively.

In an attempt to extend the results seen with immunodiffusion the soluble proteins of the days that showed precipitin bands were reconcentrated and subjected to Sephadex G-200 gel filtration. The profile is similar to that of a uterine flushing collected on day 5 post coitum, in particular for the peaks of the high molecular weight proteins and albumen. Only a tiny peak was seen in the region where uteroglobin is normally eluted (Figure 33).

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Figure 33. Sephadex G-200 chromatography profiles of the soluble proteins from a 28 day rabbit ear tissue regenerate (solid line) and from rabbit uterine flushings collected on day 5 post coitum (broken line).



A 0 day control was run. The solid line is the regenerate. The broken line is the 5 post coitum.

Figure 32. Ouchterlony double immunodiffusion. Soluble proteins from extracts of regenerating ear tissues run against uteroglobin antibody. Well six contains uteroglobin standard, Wells 1-5 contain, 0 day punch, 14 day regenerate, 28 day, 35 day, and 42 day regenerating ear tissue extracts, respectively.

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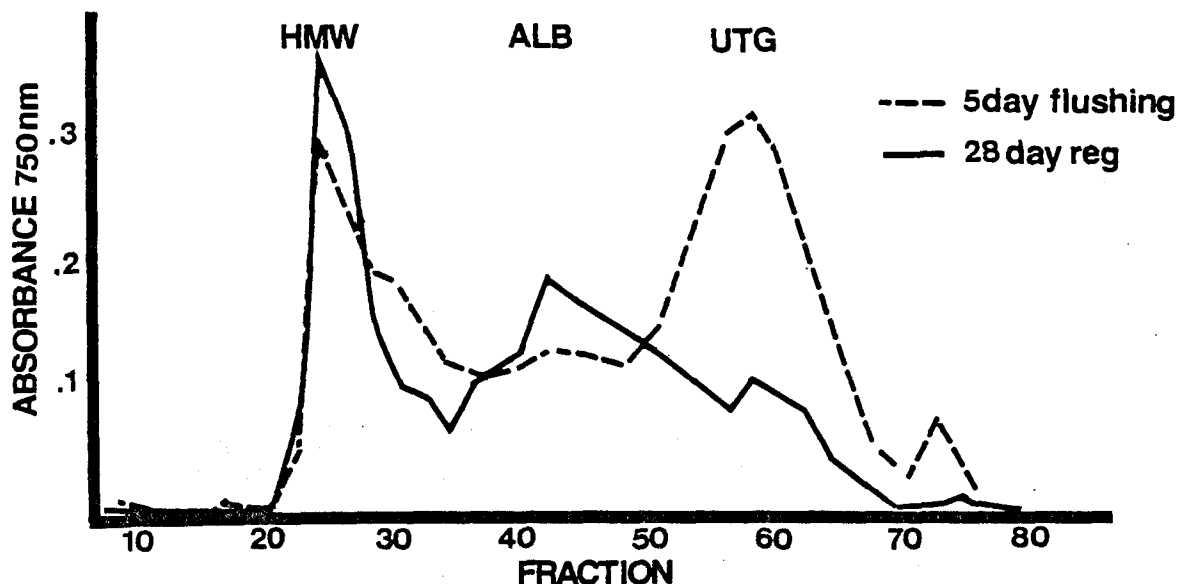


Figure 33. Sephadex G-200 chromatography profiles of the soluble proteins from a 28 day rabbit ear tissue regenerate (solid line) and from rabbit uterine flushings collected on day 5 post coitum (broken line).

A 0 day control was similar to this profile but did not contain a UTG peak. The solid line is the elution profile of the soluble proteins of a 28 day homogenate. The broken line is a typical uterine flushing profile taken on day 5 post coitum.

Non-fractionated samples were subjected to native gel electrophoresis and no band was seen to migrate in a pattern similar to uteroglobin (Figure 34). Since the major peak seen in the chromatography was albumen, a bovine serum albumen (BSA) standard was used. There are large bands that migrate in the same position as albumen in all the samples, which may reflect the blood contamination of the biopsied tissues. Other bands are seen which migrate with one of the contaminants of the BSA. Dark bands are seen at the origin in all of the samples except the standard. From our electrophoresis of the fractionated HMW proteins it is believed that this band contains those proteins which are simply too large to enter the gel. Lane 2 contains two

bands which are not contained in any of the other ear tissue samples. The second band appears to migrate in a similar position to the dimers of the albumen control. The largest quantities of soluble proteins are extracted at days 12 and 14 post punch.

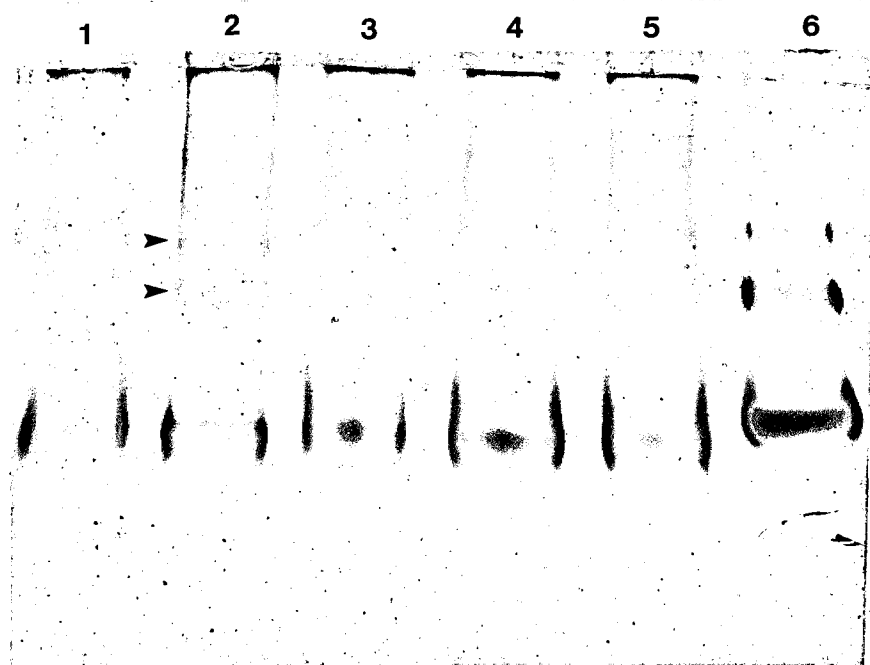


Figure 34. Native gel electrophoresis of soluble proteins from regenerating ear tissues. Lane 1--0 days after punching, Lane 2--12 days, Lane 3--28 days, Lane 4--35 days, and Lane 5--42 days, Lane 6--BSA control. Arrows--2 bands not present other days.

To determine the validity of the cross reactivity of the regenerating rabbit ear proteins and the uteroglobin antisera, the samples which gave the best bands were subjected to rocket immunoelectrophoresis. This method yields information as to the purity of the antibody and the concentration of the antigen as well as the relatedness of the antigens. Uteroglobin standards were used on either side of ear samples from day 45 post punch (Figure 35). The calculated areas under the rockets for the UTG samples match the concentration

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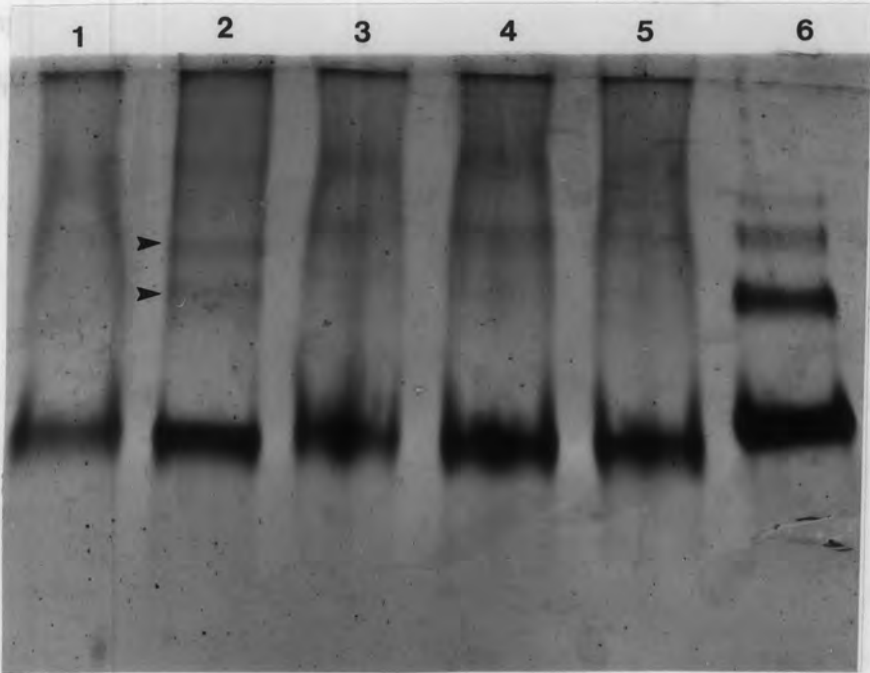


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of the proteins applied thus validating the run. No rockets are seen in the ear samples but two long streaks of some protein contaminant which reacts slightly with the antibody can be seen above each of the unknown wells extending to the gel surface (faintly obvious between wells 3 & 5). The contaminants were seen when the gels were rerun suggesting that the bands are not simply artifacts. One can also note the dark rings around the unknown wells which contain proteins too large to enter the agarose.

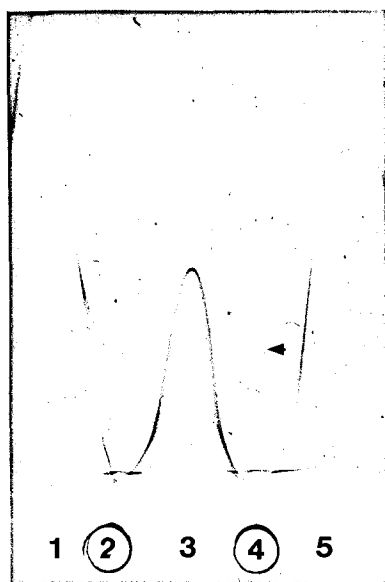


Figure 35. Pattern of migration of uteroglobin (UTG) and proteins from regenerating rabbit ear tissue 35 days after punching by rocket immunoelectrophoresis. Wells 1, 3, and 5 contain three concentrations of UTG. Wells 2 and 4 contain two concentrations of soluble ear proteins. The gel contains guinea pig anti-rabbit uteroglobin.

In view of the fact that the proteins which gave the cross reaction with the uteroglobin antibody are not uteroglobin, and the substantiation of the purity of the uteroglobin antibody it can be suggested that the unknown proteins from the regenerating tissues share antigenic determinants with UTG but are

of the proteins applied thus validating the run. No rockets are seen in the ear samples but two long streaks of some protein contaminant which reacts slightly with the antibody can be seen above each of the unknown wells extending to the gel surface (faintly obvious between wells 3 & 5). The contaminants were seen when the gels were rerun suggesting that the bands are not simply artifacts. One can also note the dark rings around the unknown wells which contain proteins too large to enter the agarose.



Figure 35. Pattern of migration of uteroglobin (UTG) and proteins from regenerating rabbit ear tissue 35 days after punching by rocket immunoelectrophoresis. Wells 1, 3, and 5 contain three concentrations of UTG. Wells 2 and 4 contain two concentrations of soluble ear proteins. The gel contains guinea pig anti-rabbit uteroglobin.

In view of the fact that the proteins which gave the cross reaction with the uteroglobin antibody are not uteroglobin, and the substantiation of the purity of the uteroglobin antibody it can be suggested that the unknown proteins from the regenerating tissues share antigenic determinants with UTG but are

not the same molecule (Appendix V). Dunbar (1977) saw a cross reaction between UTG and an antibody made against the high molecular weight (HMW) fraction of flushings collected day 5 post coitum. It was hypothesized that one of the HMW proteins might be a precursor form of uteroglobin. A HMW fraction similar to that of uterine flushings is continually observed in the regeneration tissue and evidence for uteroglobin is not seen. It may be hypothesized that the precipitin lines seen with anti-UTG are against portions of a much larger molecule with a similar structure to uteroglobin.

To test this, Ouchterlony double immunodiffusion was run with the ear proteins against HMW antisera (Figure 36) using a uteroglobin standard. Precipitin lines are seen between the ear samples from 12, 28 and 35 days post punch and the antibody. A clear precipitin band formed between the UTG standard and the HMW antisera. The band curved to meet the sample from day 12 which might indicate identity, however, the band fused with the band from day 28. The band from day 28 possesses a spur which indicates that the protein in well 28 contains additional antigenic determinants from the UTG sample. Since the UTG line does not form a spur, all the antigenic determinants of this molecule are also found in the ear antigens. We can suggest that the ear proteins that cross react with the HMW antibody are larger molecular weight molecules with additional peptides beyond those of UTG.

The last test was to show the relationship between all three groups of molecules. Ouchterlony plates were set up with ear proteins from days 12, 28, 35 and 42 post punch interspersed with standards of UTG and HMW proteins diffusing against HMW antisera. The results are seen in Figure 37. Precipitin bands are seen with components from all the wells. Wells 1 and 2 contain ear proteins from days 12 and 28 and as expected these bands fuse for identity. The bands between the 28 day regenerate and the HMW proteins also show

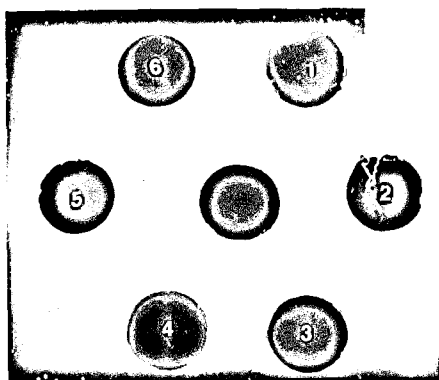


Figure 36. Ouchterlony immunodiffusion of antisera made against the high molecular weight proteins of uterine flushings from rabbits, taken on day 5 post coitum and the soluble proteins of regenerating ear tissues. Well 1--0 days post punch, well 2--12 days, post punch, Well 3--14 days post punch, Well 4--UTG standard, Well 5 --28 days post punch, Well 6--35 days post punch, Center well --HMW antisera.

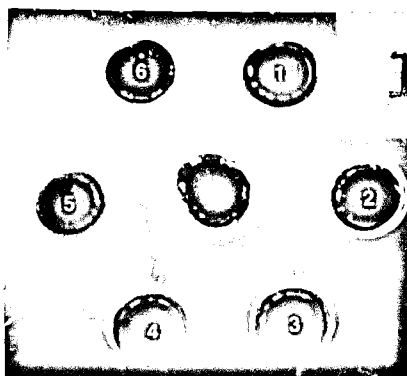


Figure 37. Ouchterlony double immunodiffusion of proteins from a flush of a rabbit uterus taken on day 5 post coitum. Well 1 contains proteins from day 12 post punch, Well 2--28 days post punch, Well 3 HMW proteins, Well 4--35 days post punch, Well 5 UTG, Well 6--42 days post punch. The center well contains HMW antisera.

identity with at least one component of the HMW complex. There are additional HMW proteins which show no identity with the ear proteins as is to be expected. The limited number of bands seen against the HMW proteins are evidence that the antibody has been adequately absorbed since in nonabsorbed samples at

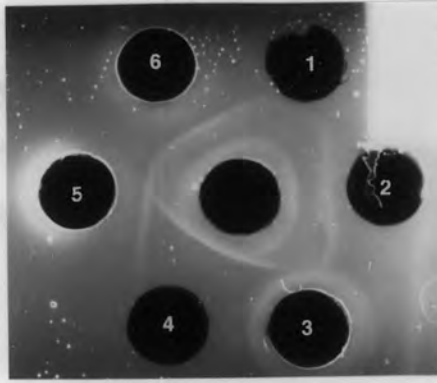


Figure 36. Ouchterlony immunodiffusion of antisera made against the high molecular weight proteins of uterine flushings from rabbits, taken on day 5 post coitum and the soluble proteins of regenerating ear tissues. Well 1--0 days post punch, well 2--12 days, post punch, Well 3--14 days post punch, Well 4--UTG standard, Well 5 --28 days post punch, Well 6--35 days post punch, Center well --HMW antisera.

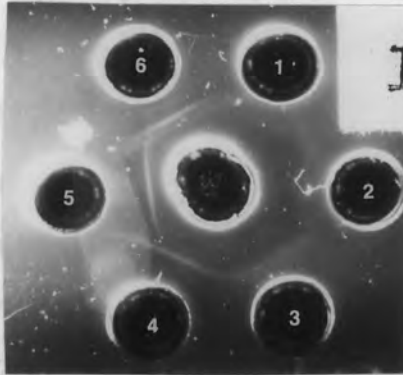


Figure 37. Ouchterlony double immunodiffusion of proteins from a flush of a rabbit uterus taken on day 5 post coitum. Well 1 contains proteins from day 12 post punch, Well 2--28 days post punch, Well 3 HMW proteins, Well 4--35 days post punch, Well 5 UTG, Well 6--42 days post punch. The center well contains HMW antisera.

identity with at least one component of the HMW complex. There are additional HMW proteins which show no identity with the ear proteins as is to be expected. The limited number of bands seen against the HMW proteins are evidence that the antibody has been adequately absorbed since in nonabsorbed samples at

least 17 bands are visible. If the gels are allowed to develop further, the band from day 35 and a component of the HMW fraction fuse for complete identity. The day 35 ear proteins and UTG do not fuse completely. Once again the ear proteins form a spur indicating similarities in the molecules but the ear proteins have additional antigenic determinants cross reacting with the HMW antisera.

Discussion

Through these studies similarities have been observed between the environment which surrounds the embryo and the regenerating tissues of the rabbit ear. In addition, in consideration of the preliminary Gallium-67 studies, we suggest that there are similarities not only between the regenerating ear tissues and embryonic or uterine tissues during early embryogenesis, but similarities also exist between regenerating tissues and tumors. Dunbar and Daniel (1979) showed that it was the macromolecular fraction of the uterine secretions which binds Gallium-67 but no effort was made to isolate the active agent. In the present study the macromolecular molecules of the ear tissue are similar antigenically to those in the uterine flushings of the preimplantation stages of pregnancy and it may well be these high molecular weight proteins which bind the Gallium-67 in the rabbit ear.

The relationship between regeneration and tumorigenesis as illustrated by the Gallium binding are intriguing. Some researchers believe that cancers result from the futile attempts of damaged tissues to regenerate (Prehn, 1971). Evidence exists which shows that tumors do not develop frequently in animals that can regenerate and that when tumors are transplanted to these animals they regress (Mattson, 1976). Prehn (1971) states that neoplasms of mammals may be the counterpart of the regeneration blastema of the amphibia, and

that the ability to regenerate is inversely correlated with the susceptibility of the tissue to tumor induction. It is interesting to note that the incidence of neoplastic disease in rabbits is very low (Weisbroth, 1974).

Our results from the biochemical comparisons of proteins in the environments of the embryo and the regenerating tissues satisfy our original hypothesis of the existence of biochemical similarities in these two systems. The role of a molecule from regenerating ear tissues that is similar to both uteroglobin and some higher molecular weight molecule of preimplantation uterine flushings may simply be to sustain the embryonic and embryonic-like cells.

Bullock (1977) reported that uteroglobin is produced in a precursor form with a larger molecular weight and Dunbar (1977) noted a reaction in immunodiffusion with antisera made against the macromolecular fraction of uterine flushings taken on day 5 post coitum and purified by gel electrophoresis. It can be suggested that the macromolecular fraction found to cross react with uteroglobin antisera may contain a larger molecular weight form of uteroglobin. Many molecules are known to exist in higher molecular weight forms and are converted to active form in the vicinity of their target tissues. Thus the existence of a molecule in a higher molecular form may indicate that it undergoes some post transcriptional processing prior to expressing its activity.

The absence of the cross-reacting molecule seen in the Ouchterlony plates in rocket immunoelectrophoresis may be related to this larger molecular weight form. Large molecules may have antigenic determinants on the surface as well as determinants hidden inside their tertiary structure. The surface antigenic determinants are readily accessible to antibody while the ones inside are only accessible after the molecules are denatured or broken apart by proteolysis. In rocket immunoelectrophoresis the proteins are not retained at

room temperature for 24 hours as are the Ouchterlony plates. One can suggest that the proteins in the rocket analysis may be closer to their native conformation. If the antigenic determinants that are similar to the uterine proteins are inside the molecule they would not be expressed, or would be expressed very slowly.

The importance of the discovery of a protein that is antigenically similar to uteroglobin in the regenerating ear tissues is suggestive that similar environments are needed for regeneration and early embryogenesis. The importance of UTG in early development is illustrated by the block seen to implantation with the administration of an antiuteroglobin antisera to pregnant animals (Krishnan, 1971; Johnson, 1974). Recently uteroglobin has been assigned an immunoblocking effect. The hypothesis was formulated by Mukherjee et al. (1980) to explain the mechanism whereby the mammalian fetus (a natural allograft) is not rejected by the mother in early development. They suggest that uteroglobin crosslinks (via the enzyme transglutaminase) with Beta₂-microglobulin on the embryonic cell surface forming a complex which masks the H-2 and H-L-A antigens of the implanting embryo. Recent reports of experimental data, currently in press, suggest that this hypothesis is gaining credibility.

In view of the recent evidence for the role of uteroglobin as an immunoblocking agent, it is challenging to speculate as to its possible role in ear tissue regeneration. If the tissues of the ear dedifferentiate both morphologically and physiologically, does this morphological change include the loss of specific cell surface antigens needed for self-recognition? With the loss of these antigens an immune suppressive molecule such as uteroglobin would be needed, yet no evidence exists for such a change in these tissues. A

controversy exists over the capacity of tumor cells to alter their cell surface antigens with transformation. Warner (1980) cites evidence that tumor cells in culture do change the antigenic determinants on the cell surface and will elicit an immune response from isolympocytes. An additional explanation might be that with the vast changes in tissue structure and remodeling, sequestered antigens may be exposed, and to prevent cell destruction, an immune suppressive molecule would be needed.

This study has provided informaton which supports our original hypothesis, namely that biochemical similarities do exist in the environments of regeneration and early embryogenesis. This is important since UTG possesses such a unique molecular structure. UTG is unique to the rabbit yet proteins which are analogous to UTG temporally or chemically, either in molecular weight or in electrophoretic mobililty, have been found in other species (Daniel, 1976). However none of these proteins have been shown to have a similar molecular structure. In a cross-reference of protein molecular structures the only protein which was similar to UTG was promelletin from honey bee venom, and then the similar portion was limited to a seven amino acid sequence. Thus the antigenic similarity between UTG and a component from the regenerating ear tissues is significant and unusual. Collectively our studies have shown a number of biochemical similarities with embryogenesis; transitions in the types of collagen, similar to those changes seen in culture or embryonic systems; the presence of two embryonic collagen types; similarities in collagen levels and collagenase; and the presence of a protein similar antigenically but apparently containing additional determinants.

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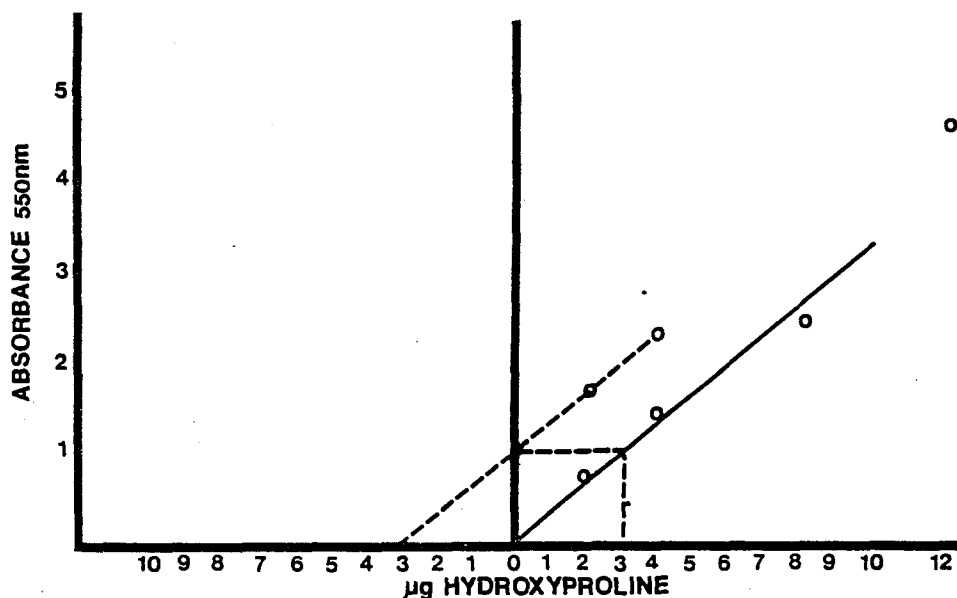
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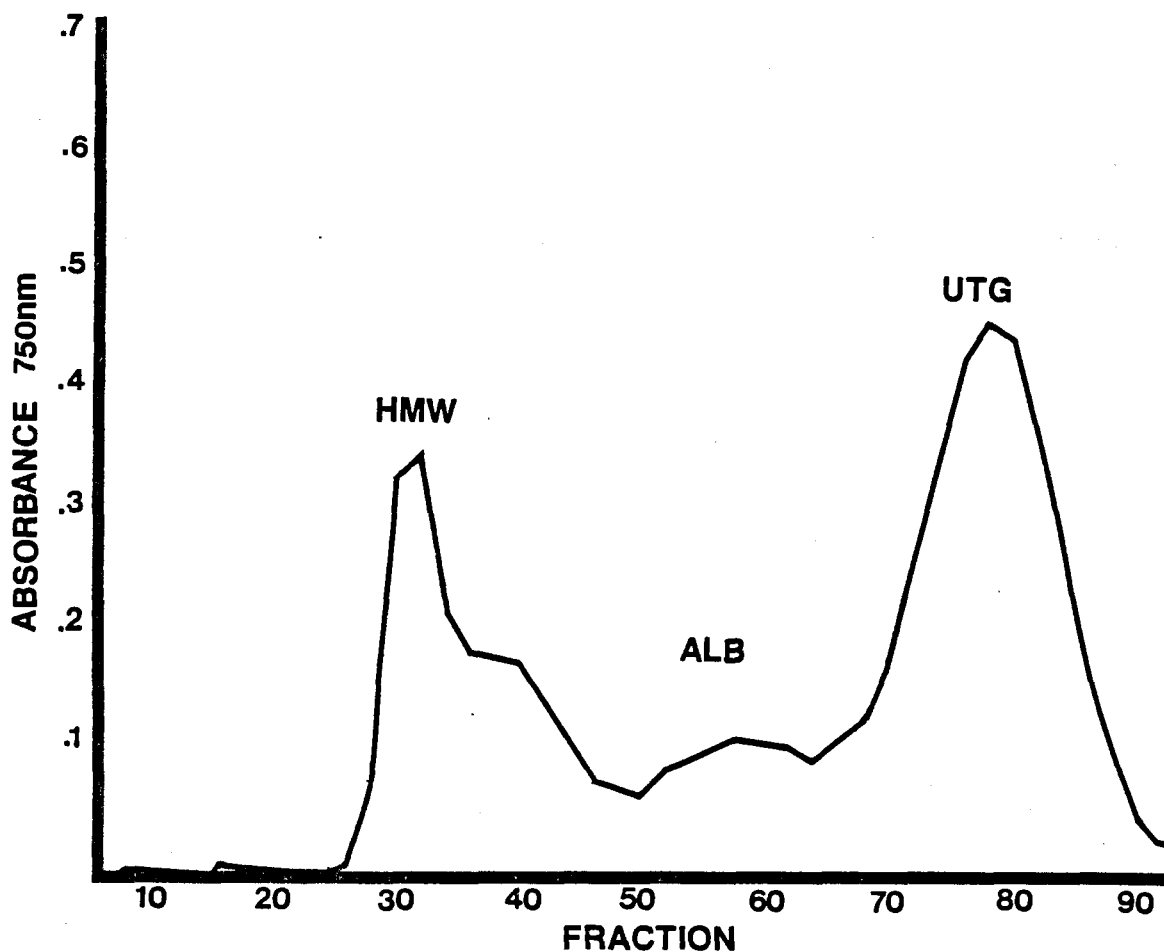
APPENDICES



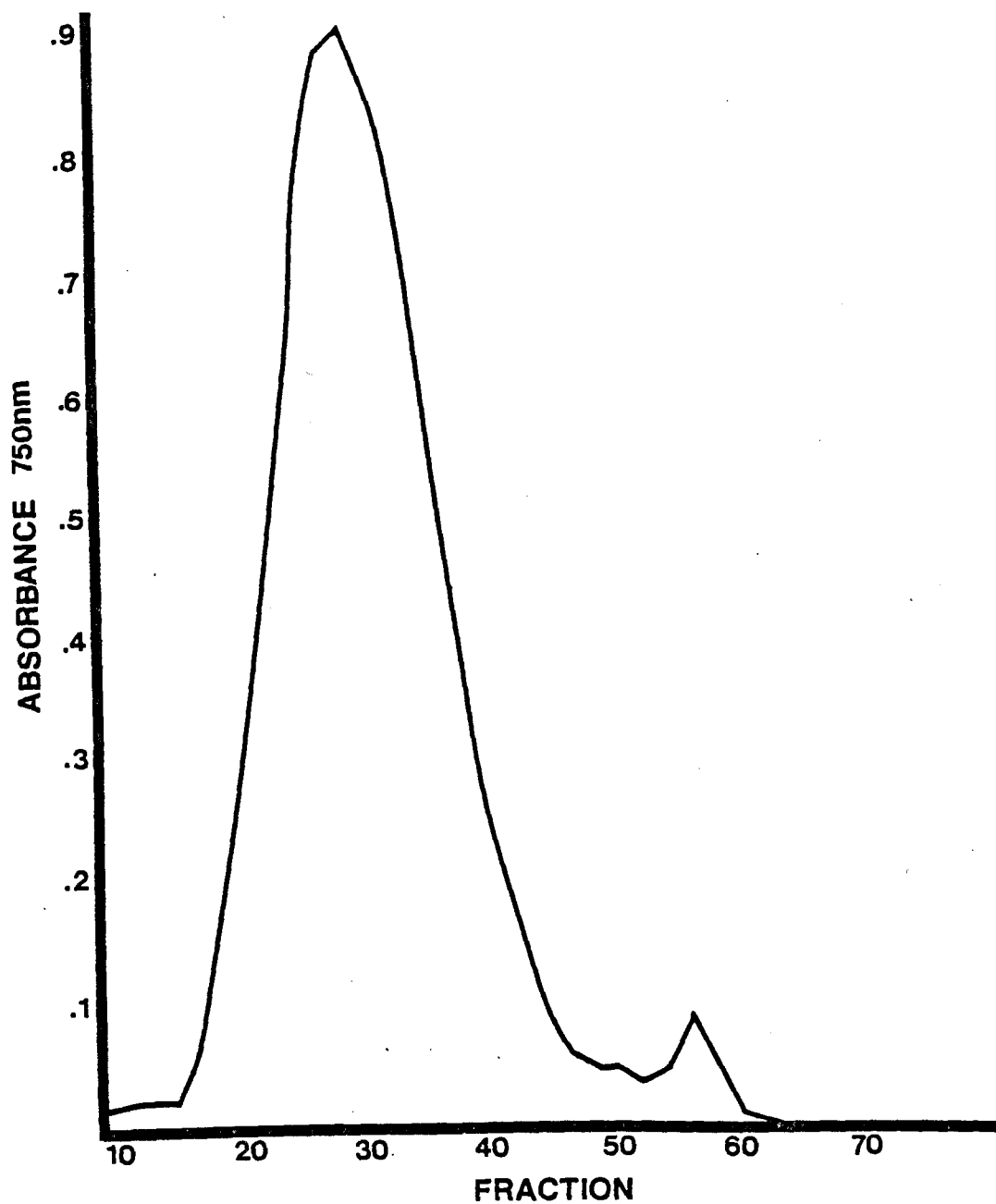
Appendix 1. Standard curve of hydroxyproline, solid line. Duplicates were run for each concentration. The broken line shows the assay with the standard addition technique. The y-intercept is the unknown sample with no added standard. By making a linear plot of the unknown, and unknown plus standards, the x-intercept becomes the unknown quantity. Additionally a linear regression was performed on the unknown points. By all three methods the results were in close agreement.

Standard curve
X-intercept
Linear regression

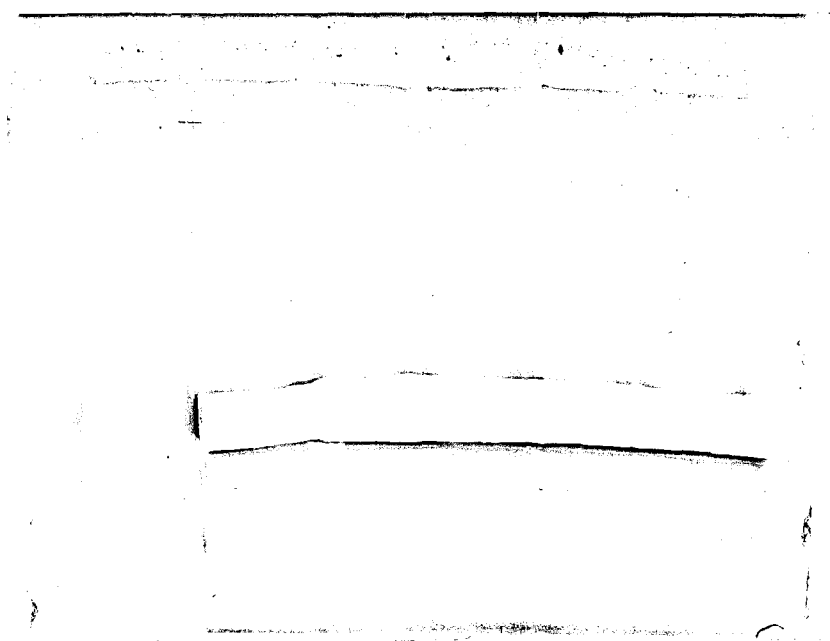
Unknown Sample
3.1 μg hydroxyproline
3.2 μg hydroxyproline
3.06 μg hydroxyproline



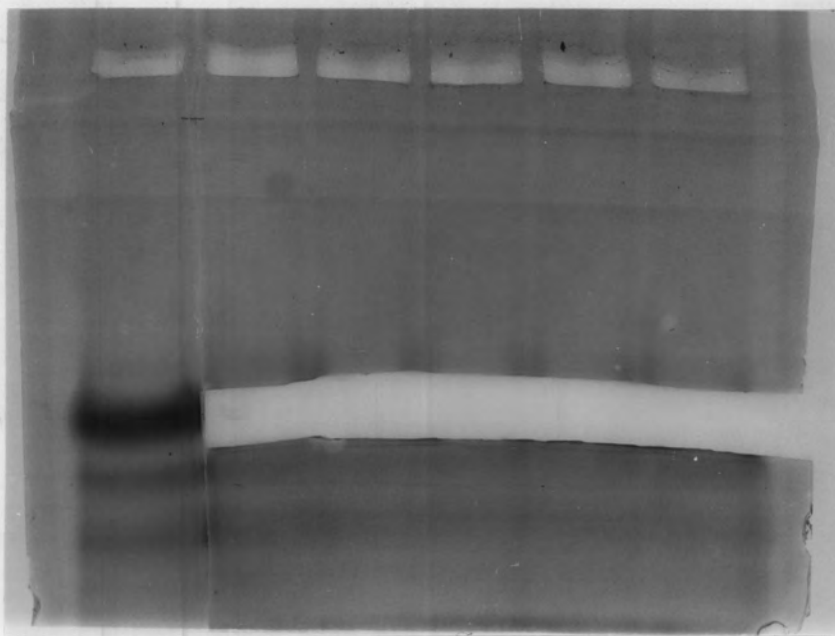
Appendix II. Sephadex-G-200 chromatography profile of rabbit uterine flushings taken on day 5 post coitum. Three major peaks are obvious. The uteroglobin (UTG) peak was retained for antibody production.



Appendix III. Sephadex G-50 chromatography profile of the uteroglobin fraction eluted from Sephadex G-200.



Appendix IV. Native gel electrophoresis of the uteroglobin fraction from 5 day uterine flushings eluted from Sephadex-G-50. The slice removed from the gel contains only the darkly staining band and was used to immunize guinea pigs for antibody production.



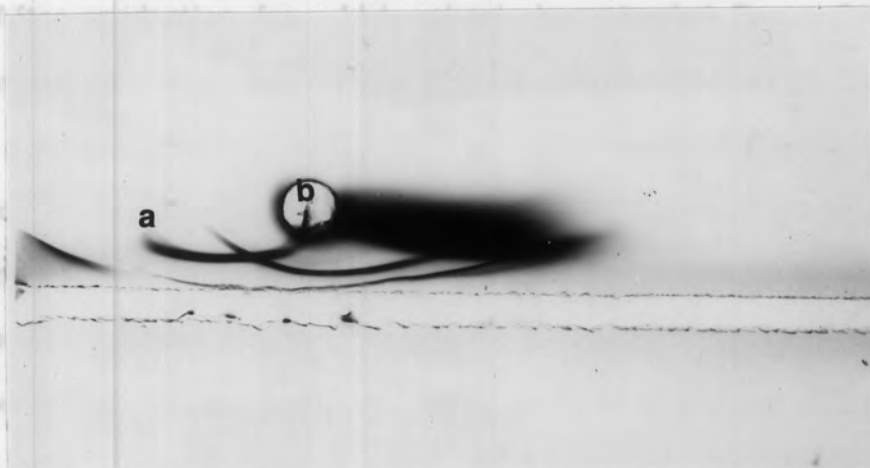
Appendix IV. Native gel electrophoresis of the uteroglobin fraction from 5 day uterine flushings eluted from Sephadex-G-50. The slice removed from the gel contains only the darkly staining band and was used to immunize guinea pigs for antibody production.



Appendix V. Tandem electrophoresis of uteroglobin and high molecular weight (HMW) proteins from uterine flushings taken on day 5 post coitum against high molecular weight antisera. Well a-uteroglobin, well b-high molecular weight proteins. Two bands are seen with the uteroglobin antigen, one which migrates immediately below the sample well and shows no identity with the similarly migrating component of the HMW proteins. A second component of the UTG sample fuses with the similarly migrating component of the HMW proteins. It can be suggested that either the first band is a breakdown product of the band near the trough, or the band at the trough is a larger molecular weight precursor of the band at the well.

VITA

Patsy Kyline Williams-Boyce was born in Burnsville, North Carolina on April 28, 1952. She received a portion of her elementary education in North Carolina, with the majority at McDonald School in Mohawk, Tennessee. She attended Cocke County High School in Newport, Tennessee and was graduated in 1970.



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In September 1978 she entered the doctoral program in the Department of Zoology at the University of Tennessee. While conducting her research, she was supported by a departmental teaching assistantship. She was awarded the Doctor of Philosophy degree with a major in Zoology in August, 1982. She is married to Stephen L. Boyce.