Augmentation of Regenerative Healing in Man

A Possible Alternative to Prosthetic Implantation

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"More reliance should be placed upon the primordial power of the human skeleton to regenerate injured and missing substance."†

The present thrust of clinical investigation in orthopedic surgery is towards the replacement of missing or damaged skeletal parts with metallic or plastic implants. One can only agree that major advances have recently been made in this direction and that at this time such procedures are frequently the best method of therapy for many pathological conditions of the skeletal systems. Despite their popularity and demonstrated efficiency, the use of such devices fails to take into consideration two basic facts: first, the human skeletal system is capable of considerable self-repair; second, no inorganic implant has the capacity of growth and remodeling and can only decline in mechanical strength with the passage of time. The statement that the best replacement for a damaged femoral head would be a new structure regenerated by the individual himself still remains unchallenged. Is it possible to enhance the reparative ability of human bone so that such events could occur? Would any technic devised for this purpose have a wider therapeutic role such as the acceleration of tissue healing in general? Is it within the realm of possibility to restore to the human the capacity for regrowth of multi-tissue appendages? A sequence of events begun in 1945 indicates that the answers to some of these questions may soon be in the affirmative.

It would appear desirable that clinicians be informed of the status of research in this rapidly advancing field in order to have the necessary consensus and enthusiasm for clinical investigations when they become warranted. This paper is therefore an attempt to briefly review the regenerative process as it is currently understood, and to integrate the significant advances that have been made since 1945 into a new unified theory which may furnish the framework for clinical studies.

Regeneration is the complete replacement of lost body parts by cellular growth, with the end product morphologically and functionally indistinguishable from the normal. Many invertebrates have this ability to a high degree and are capable of regenerating more than 50 per cent of their total body mass.[1,2] Among the vertebrates, the process is somewhat more limited, with perhaps the most outstanding example being total limb regeneration in the salamander (in which a structure of complexity equal to a human appendage is regenerated with apparent

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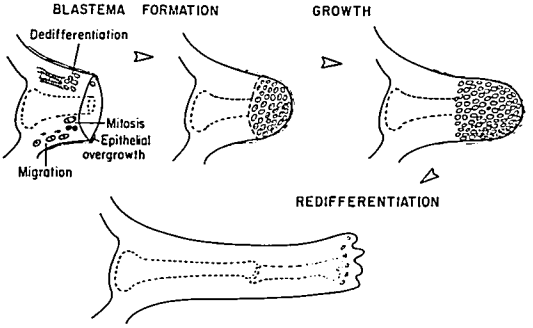

FIG. 1. Cellular events in regeneration. The limb regenerative process may be rather arbitrarily divided into 2 stages, both of which are cellular in nature. The first is the accumulation of the cell mass or blastema which may be derived from several sources, but is nevertheless characterized by the primitive undifferentiated nature of the component cells. The second stage is characterized by redifferentiation and growth via mitotic activity. In this phase the necessary structures appear in the required places. It would appear logical that the two stages need not be governed by the same control system.

ease). In man, regeneration is limited primarily to bone, where fracture healing is a true regenerative growth process.[18] Compensatory regrowth of skin and portions of the liver and other parencymatous organs does occur, but it represents an acceleration of a normal replacement rate and lacks both the competency and specific cellular phenomena of true regeneration. Neural tissue displays even less competency, "regeneration" of peripheral neurones being no more than the regrowth of a specialized appendage of a single cell.

The essential portion of the cellular events (Fig. 1) in regenerative healing is the accumulation of primitive, undifferentiated cells at the site of the trauma. This cell mass, called the blastema, is produced by either migration of specific undifferentiated cells, dedifferentiation of mature cells in the surrounding surviving tissue, or proliferation of a specific stem cell line. The blastema subsequently undergoes mitotic activity and forms a replica of the missing part with all original tissues replaced in correct position and functionally restored.

In 1945 Rose[24] reported on a series of experiments in which he was able to demonstrate some limb regeneration in adult frogs (contrary to popular opinion, a species not capable of this process) by immersion of the animal at frequent intervals in half-saturated NaCl. (Polezhayev[20] had earlier induced some limb regeneration by trauma in larval frogs which had just lost regenerative ability; nevertheless, the first clear demonstration of induction of regeneration in an adult form not normally capable of this process was by Rose as cited above.) A recognizable blastema formed beneath the epithelium from dedifferentiation of some of the surviving mature tissues. Rose attributed the result to the treatment preventing complete epithelialization of the amputation stump; however, the alternative explanation that the saline treatments constituted repeated trauma to the amputation stump had to be considered. In 1946 Polezhayev published results[21] substantiating this latter viewpoint by demonstrating similar amounts of limb regeneration in frogs, produced simply by repeated mechanical trauma to the amputation stump. These were crucial observations from a number of viewpoints. Firstly, they indicated that trauma, rather than being a nonspecific event, was in fact, a specific stimulus with a quantitative threshold value necessary to initiate the

process of regeneration. Secondly, they demonstrated that under experimental conditions some measure of regeneration could be restored to certain animals normally lacking this ability. In this fashion the study of regeneration passed from the observational to the experimental.

In 1952 Singer[28] brought together many observations on the crucial relationship between the innervation of a limb and its regenerative capacity. He was able to demonstrate a threshold value for the mathematical ratio between the amount of nerve tissues in an extremity and the total extremity tissues which, if exceeded, enabled regeneration to occur. The effect has been used to restore limb regeneration to animals normally lacking this capability with Singer himself,[29] demonstrating some limb regeneration in the adult frog by surgically augmenting the normal nerve supply in sufficient amounts to satisfy the threshold mass ratio. More recently, Mizell[19] has utilized a similar technic on newborn opossums and again demonstrated unquestionable digital regeneration in this primitive mammal. The mechanism responsible for this specific effect of the central nervous system on regeneration remains unexplained. Singer[17] has recently demonstrated that extracts of peripheral neurones enhance protein synthesis in the denervated limb; they, however, were unable to restore any regenerative capacity to such a limb.

Also in the early 1950's Schotté,[25] reporting on a long series of experiments, noted that hypophysectomy prevented limb regeneration in animals normally capable of this process. Subsequently in 1958 he was able to induce some measure of limb regeneration in frogs by surgically implanting additional adrenal tissue.[26] At that time however, it was not evident which hormones were specifically involved nor what their mode of action was.

In 1961 I reported[5] that the electrical potentials at the site of limb amputation in a regenerating salamander followed a much different pattern of change during the course of this type of healing than during simple healing at a similar site in the closely related but non-regenerating frog. While the initial electrical potentials in both forms were positive in polarity and very similar in magnitude, the salamander subsequently demonstrated a polarity reversal, with the stump becoming highly negative, reaching a maximum during the appearance of the blastema. The frog, on the other hand, demonstrated only a slow return of the positive polarity to normal levels as healing by epithelialization and cicatrization took place. On the basis of prior work,[4] the potentials were attributed to an interaction between the local current of injury and a direct current activity of the central nervous system. Subsequent investigations of this DC neural activity demonstrated that it constituted an organized data transmission and control system that was directly concerned with the sensing of trauma and the subsequent repair processes in addition to several other similarly primitive modalities.[6] It was therefore postulated that this electrical activity could be equated to Singer's neural factor and that simulation of the appropriate electrical environment in an amputation stump of a non-regenerating animal should produce some measure of limb regeneration. This was recently confirmed by Smith[30] who produced limb regeneration in frogs by implanting bi-metallic electrogenic devices in the amputation stump. Blastema formation and regeneration regularly occurred when the device was implanted to produce a relative electrical negativity at the amputation stump and was either absent or reduced in extent with the opposite orientation. While the exact mechanism of action of the electrical potentials in producing this limb regeneration was not then evident, we developed the thesis that the quantitative relationships of trauma and innervation to regeneration were both expressions of a necessary threshold of electrical phenomena.

In the interim we became interested in

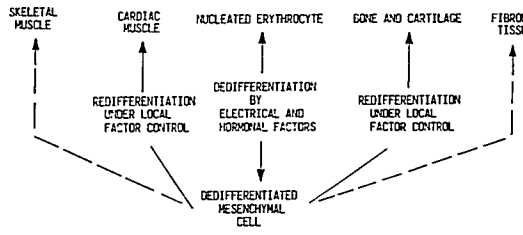

FIG. 2. This sequence is based upon our observations on the amphibian nucleated erythrocyte. The solid lines are cell transformations that have been observed either in vitro or in vivo, the dotted lines indicate predicted transformations which appear to have a high probability. The local factors governing redifferentiation are currently unknown, but are postulated to be either chemical or local cell surface contact phenomena.

the fracture healing process since this appeared to constitute an exception to Singer's neural requirement for regeneration. The healing of a fracture, in mammals, follows a histologic course that is typical of a regenerative process complete with the appearance of a blastema derived, in part, from the mitotic activity of periosteal and endosteal stem cells[31] despite the fact that bone is very poorly innervated[27] and, according to Singer's hypothesis, should not be capable of regeneration. We were able to demonstrate that bone, when mechanically stressed, produces electrical potentials[2] and that simulation of these electrical phenomena by implanted battery powered devices in dogs could produce marked proliferation of the endosteum at the negative electrode site.[3] The level of current necessary to produce a marked mitotic response was very small with an effective range of 2 to 10 μA. It was therefore concluded that bone growth in response to stress was the result of the electrical stimulation of mitotic activity and that the source of the electrical phenomena was the electrogenic property of the bone matrix itself.[7]

Subsequently, in a study of fracture healing in the Amphibia, we discovered that the source of the regeneration blastema in this case was dedifferentiation of the nucleated erythrocytes in the fracture hematoma and that this dedifferentiation could be produced by the in vitro exposure of normal nucleated erythrocytes to extraordinarily low levels of electrical current equivalent in magnitude to those produced by the bone itself at the fracture site in vivo.[8] This process was investigated in detail at the cellular level[9] and we have been able to determine that the mechanism of action of the electrical current is via a membrane-mediated effect upon the RNA-DNA apparatus. More recently this same target cell, the nucleated erythrocyte, has been found to be directly involved in myocardial regeneration in Amphibia,[10] and investigations are currently underway to determine its contribution to limb regeneration in this genus. It must be noted at this point that these observations can only be interpreted as indicating that the original dedifferentiation process involves a return to essentially the primitive mesenchymal cell type with the cell subsequently capable of expressing any mesenchymal genome in its redifferentiation process. The exact genome expressed is dependent only upon local factors, i.e., the necessary instructions being furnished by the surviving local tissues in some fashion (Fig. 2).

In the course of these investigations we noted that during the winter months the erythrocytes of the frog became refractory to the influence of electrical currents except in the case of females who were actively engaged in egg production during this period. In fact, at a certain time in this process, the red cells of the female became more sensitized to the effects of the current and were capable of full dedifferentiation at current

levels 300 to 500 times less than the normal effective level in the summer months. This gave the appearance of a specific hormonal sensitization of the target cell population. The question of which hormone was involved appears to have been recently resolved. Thornton and his associates, working with the hypophysectomized newt, have demonstrated quite conclusively that the hormone necessary to restore limb regeneration to this preparation is prolactin.[12] This hormone has been found in the pituitary gland of all classes of vertebrates and performs several important functions in the reproductive cycle[15]; it is therefore postulated that it is the agent responsible for the enhancement of the electrical effects on the female RBC's during oogenesis. The only remaining question is the site of action of this hormone; i.e., is it an effect directly upon the target cell itself, or is it a general metabolic effect? We have found that exposure of nonreactive amphibian erythrocytes to very low concentrations of prolactin restores their ability to dedifferentiate in response to low level electrical factors. It is postulated that this is via a sensitizing effect upon the cell membrane similar to that noted by Ballard and Tompkins.[1]

Our success in stimulating mitotic activity in mammalian endosteum and periosteum and dedifferentiation in amphibian erythrocytes, all by very low levels of electrical current led us to evaluate the effect of similar low levels of current on mammalian epidermal cells. Gelfant[13] had previously shown that trauma was a stimulus to epidermal mitotic activity in the mammal and, utilizing his preparations and technics, we obtained preliminary evidence for electrical current (of correspondingly low amplitude and unaccompanied by trauma) producing similar mitotic activity in mammalian epidermal cells.

Several things become apparent on reviewing all of these data; both trauma and innervation play an important role in the regenerative process, apparently via a final common pathway of electrical charge, the hormone prolactin appears to sensitize appropriate cells to the action of this agent, and finally limb regeneration has been restored to a variety of lower vertebrates by several methodologies, all related to the foregoing. At this point it became possible to integrate these data into a theoretical control system initiating and regulating regeneration (Fig. 3) and it appeared appropriate to assess the position of the Mammalia in this framework.

Aside from the acquisitions of temperature control and loss of red cell nuclei, an outstanding difference exhibited by the Mammalia compared to the remainder of the vertebrates is the marked tendency towards encephalization in the CNS, which results in major deficiencies in the nerve mass to total tissue mass in the extremities. It has been reported that the magnitude of the current of injury is directly proportional to the amount of muscle innervation,[33] and it may therefore be theorized that the unfavorable nerve mass to limb mass in Mammalia results in a failure to generate the minimum electrical factors necessary to bring about dedifferentiation of appropriate cells at the site. A corollary of this is obvious—have the cells of the mammals lost the ability to dedifferentiate under appropriate circumstances? Gillman and Wright[14] have presented some evidence indicating the dedifferentiation into fibrocartilagenous cells in the mammalian fracture callus. The number of such cells available, however, would be manifestly inadequate to furnish any respectable blastema. Within recent years a peculiar property of the mammalian lymphocyte has been reported. Exposure of mature, small, circulating lymphocytes to phytohaemagglutinin (PHA) causes them to undergo a transformation into a morphologically more primitive form (originally termed "blastoid") which is subsequently followed by one cell division.[23] More recently, the ensuing transformation has become generally considered to be true dedifferentiation.[22] There are reasonably large numbers of circulating lymphocytes, and the

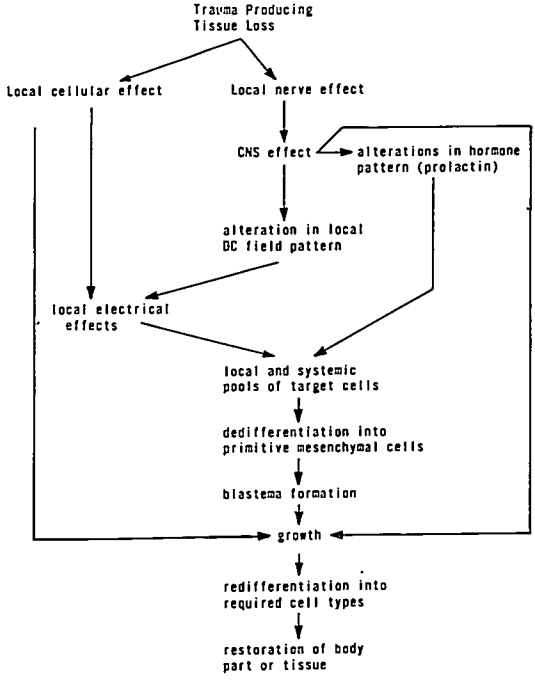

FIG. 3. Theoretical schematic of control system regulating regenerative type healing. The basic premises required for this control system are: (1) the neural factor of Singer is electrical in nature; (2) the function of the electrical factors is to produce dedifferentiation of a pool of suitable "target cells"; and (3) the hormone prolactin sensitized the target cells to these electrical factors. In theory then, an organism that does not regenerate may lack either adequate electrical potentials at the site of injury due to inadequate innervation, adequate pools of "target cells," or adequate levels of prolactin. If there is an adequate pool of "target cells" then regeneration could be induced by artificially augmenting electrical and hormonal factors to adequate levels.

lymphoid tissues are widespread and capable of producing additional numbers of these cells. Hence, we determined the effect of comparable electrical and hormonal factors on these cells.

First, we exposed amphibian nucleated erythrocytes to PHA, in vitro, with resulting morphological changes identical to those produced by electrical currents. However, the efficiency of the process (as judged by the speed of the response and the percentage of cells involved) was approximately 50 per cent of that resulting from exposure to maximally effective electrical parameters. We next exposed human lymphocytes (obtained by dextran separation) to the optimal current levels previously noted for the amphibian erythrocyte. Morphological changes identical to dedifferentiation ensued. It was later determined that exposure to very small concentrations of prolactin appeared to enhance the morphological response to the electrical current. These two cells then, the human lymphocyte and the amphibian nucleated erythrocyte, are remarkably similar in their responses to PHA, electrical current and the hormone prolactin. In the Mammalia then we have one cell type (the lymphocyte) that appears to be dedifferentiated by exceedingly small electrical currents, and two cell lines (epithelial and periosteal) that can be similarly stimulated into mitotic activity. It would therefore appear that all mammalian cells have

not lost their ability to respond appropriately to suitable levels of electrical current and to have this effect enhanced by exposure to low level concentrations of appropriate hormones. Therefore, the defect that precludes regeneration in the higher vertebrates is theorized to be primarily the inability to generate threshold electrical phenomena in response to trauma with a subsequent failure to provide an adequate cellular base for blastema formation. It is evidently well within the current state of the art to supply an adequate electrical environment to a traumatized site and to ensure an adequate level of the sensitizing hormone.

Thus far our technics have been applied to only a few mammalian cell lines with dedifferentiation apparently occurring in only one of them, the lymphocyte. This has to be contrasted to the situation in the salamander where the circulating erythrocyte is available in large numbers at the site of trauma and in addition the skeletal muscle is also capable of dedifferentiation.[16] Therefore, unless additional cell lines in the mammal are capable of being dedifferentiated by a combination of electrical and hormonal factors, supplying these factors in adequate amounts will not result in an adequate blastema. Experiments are currently underway to determine whether mammalian skeletal muscle can undergo the necessary transformation; however, there are several other possibilities that should be considered. It may be possible to stimulate an increase in the total number of circulating lymphocytes, or perhaps even better, to produce a large number of circulating erythrocytes still possessing their nuclei. In the latter case, while experiments to determine the capability of such cells to dedifferentiate and take part in regeneration have not been completed, the clinical observation of the rapid healing of fractures in bones containing hematopoietic marrow would seem to indicate that this is a distinct possibility. In addition, Boyne[11] has recently demonstrated the in vitro ability of hematopoietic marrow to transform into bone when stimulated by exposure to a small fragment of cancellous bone.

Should appropriate cellular populations be available in the human and should the simulation of the necessary electrical and hormonal factors result in an adequate blastema, the problem of the data transmission necessary to form the desired structure would appear to be much less formidable than previously considered. The programming of the dedifferentiated cell to express the required genome in its subsequent redifferentiation is, at least in part, dependent upon local factors, similar to a self organizing system, and it is possible that the residual extremity nerve supply in the mammal would be adequate for any necessary remainder.

It must be emphasized that much more experimental work remains to be done before these technics can be safely applied to humans. Nonetheless, the outlook at this time appears to be more hopeful than ever before for the eventual restoration of this "useful disposition"‡ for the regeneration of missing or damaged body parts to man.

SUMMARY

The possibility of stimulating the naturally occurring ability of bone and associated tissues to regenerative healing is the alternative to inorganic prosthetic replacement. A brief review of research done on regenerative systems over the past 25 years including a summary of the latest findings implicates bioelectric phenomena.

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‡ Spallanzini (Prodromo, 1768).

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