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Regulation of Growth of Fibroblasts

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Present knowledge on regulation of fibroblast growth is based on in vitro culture of fibroblasts from different sources. The research has focused on 2 problems: identification of the signal that reaches the fibroblast from outside and tells it to grow and identification of the metabolic reactions inside the cell that commit it to initiate DNA synthesis after the signal arrives. Although the signal and the metabolic reactions have not yet been clearly identified, and the relationship between in vivo conditions and the result of these in vitro studies still has to be determined, the large body of data collected so far and the steadily growing information concerning these problems suggest a complex interrelation between cellular environment and metabolic processes involved in growth regulation.

Our knowledge of growth regulation of fibroblasts derives more from interest in growth regulation of vertebrate cells in general than from special interests in fibroblasts. One has to keep this fact in mind because some questions of special interest to dermatologists have not been asked by investigators with a broader interest in general growth regulation.

Investigators have chosen to work with fibroblasts because the cells are readily available for tissue culture and grow easily and preferentially from explants or digests of most tissues and organs. Fibroblasts grow relatively fast and can be harvested in large quantities. Continuous cell lines can be established from the explanted cultures, and they can be transformed by carcinogens or tumor viruses in vitro for use in studying the properties of tumor cells.

The following discussion presents an overview of results obtained from tissue studies of cultures of "normal" fibroblasts. Although in some cultures, such as those from embryonic sources, the cells may have a different mesodermal origin, such as chondroblasts or osteoblasts, it is accepted by most cell biologists that the cells used in the investigations described below are fibroblasts.

FIBROBLASTS IN CULTURE

The cells normally are taken from human biopsy specimens or from chicken, mouse, rat, or hamster embryos. Fibroblasts derived from biopsies have only a limited in vitro life span; it varies from approximately 20 to 60 doublings according to the age of the donor [1]. Studies with such cultures can be carried out for several passages before the cells are no longer suitable for growth in tissue culture; sometimes one can select from these cultures cell lines that can be propagated indefinitely. One such continuous line in wide use is the 3T3 cell line established by Todaro and Green [2] from mouse embryonic fibroblasts.

When normal untransformed fibroblasts are transferred to plates, usually in a defined nutrient medium supplemented with serum, they grow logarithmically until they reach their saturation density, which is determined by the cell type, culture medium, and serum concentration. Often culture conditions are such that cells stop growing soon after they reach confluency. Early observations of cessation of cell growth as cells reached confluency prompted investigators to suggest that cell-to-cell contacts are the signal that halts growth. Vogt and Rubin introduced the term "contact inhibition" of growth for this phenomenon [3]. However, initial plating of the cells in higher concentrations of serum resulted in growth to higher cell densities [4]. The conclusion that the authors have drawn from this fact is that the observed growth arrest is due to the availability of growth factors in the culture medium and the availability of surface area rather than to establishment of contacts between cells. We now prefer to use the term "density-dependent regulation" of growth instead of "contact inhibition" to describe this phenomenon.

Arrest can also be achieved by a change from medium with serum to medium without serum. The arrested cells are usually in the $G_1(G_0)$ stage of the cell cycle (Fig 1). Growth of the cells can be initiated by the addition of serum to the medium; growth then will proceed through the cell cycle in a relatively synchronous fashion (Fig 2). Ten to 15 hr after the addition of serum, DNA synthesis begins; mitosis occurs from 24 to 30 hr after serum addition. The amount of time required to enter the S phase varies somewhat from one cell type to another. In cultures stimulated in the resting phase, the $G_1(G_0)$ period is usually greatly elongated compared to logarithmically growing cells. Virally or chemically transformed fibroblasts usually do not stop growing under normal culture conditions, have a much lower serum requirement, and may become arrested throughout the cell cycle.

Since normal untransformed fibroblasts in culture seem to show the same behavior as their counterparts in vivo, i.e., they remain in $G_1(G_0)$ when they are in a nonproliferating state, research has focused mainly on the following questions: "What are the factors stimulating the cells to grow?" "What are the early events that take place inside the cell after it has received the signal to grow?" and "Which of these events commit the cell to synthesize DNA 10 to 15 hr later?"

GROWTH FACTORS

Several groups have partially purified or isolated growth-stimulating activities from serum or tissue. Some of the growth factors, e.g., insulin, were already known to have activity not related to growth. A list of growth factors for stimulating normal fibroblasts arrested in $G_1(G_0)$ is given in the Table. Of the last 5 substances in the table, hydrocortisone may be especially interesting to clinicians who frequently use this substance to stop the growth of fibroblasts and other cells. Growth stimulation of fibroblasts by hydrocortisone first reported by Thrash and Cunningham [34], may not occur in all fibroblasts in culture; some groups have found that it is not active alone but rather potentiates the effect of other growth factors [20].

The factors listed in the Table do not produce as great a stimulatory activity as serum when added to cultures whose growth was arrested in G_1. A background of serum, usually 0.1 to 0.5%, which by itself is not stimulatory, has been used for optimal stimulation with purified growth factors and for maintenance of quiescent cultures in a healthy state. It is apparent

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Abbreviation:
EGF: epidermal growth factor

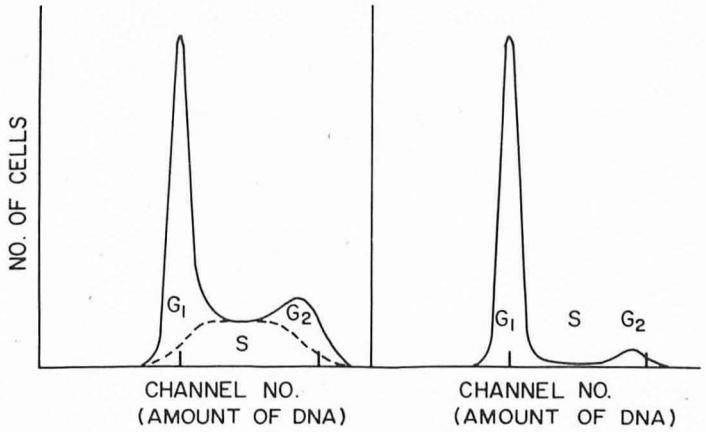

FIG 1. Analyses, obtained with a Los Alamos design flow microfluorometer, showing DNA content per cell in: (*left*) a rapidly growing 3T3 cell population with approximately 40% of the cells in S phase and (*right*) a quiescent 3T3 cell population in which almost all of the cells have the G_1 content of DNA. Graphic estimations of the percentages of cells in G_1, S, and G_2 + M were based on the height of the curve in the middle of S, as is illustrated by the *broken line* in the analysis of the growing cell population.

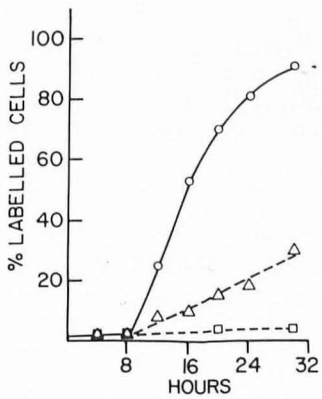

FIG 2. Time course of initiation of DNA synthesis, shown by autoradiography (radioactive thymidine incorporation from time zero), in sparse quiescent 3T3 cells after the addition of: ○, 4% serum; △, 1% serum. The controls (□) remained in 0.2% serum.

that some of the growth factors are much more active in certain combinations than individually. With a low background of serum and the appropriate combinations of factors, stimulation nearly equivalent to that achieved by the addition of fresh serum can be obtained. An example of this is the stimulation of 3T3 cells by fibroblast growth factor (FGF), insulin, dexamethasone, and an ammonium sulfate fraction from serum (Fig 3) [20].

EPIDERMAL GROWTH FACTOR

A growth factor of polypeptide nature is generally thought to exert its initial effect on the cell by interacting with the cell surface, specifically by binding to a receptor on the cell membrane. Several groups have investigated the binding of peptide hormones, especially insulin, to the cell surface. However, in mammalian cells the level of growth activity produced with insulin is rather low, and it seems that its role is more of a supporting one for other growth factors. Because purified epidermal growth factor (EGF) is active not only on epidermal tissue but also on cultured human fibroblasts [6] and because it is available in ^{125}I-labeled form, it has been used in the study of the biochemical events involved in the binding of a polypeptide growth factor to cultured fibroblasts.

EGF was first isolated from extracts of the male mouse submaxillary gland in 1962 and described by Cohen [38]. Its biological activity was assayed by precocious opening of the eyelids and eruption of the incisors in newborn mice. Human EGF was recently isolated from the urine of pregnant women [39].

The biological properties of human EGF have been examined by Carpenter and Cohen [40]. They found that EGF is active in extremely low concentrations. It binds rapidly and reversibly to specific binding sites on the human fibroblast cell membrane. The concentration required for maximal binding ($1.5\text{-}2 \times 10^{-9}$ M) is 4 times greater than that required for maximal stimulation

Factors stimulating growth in cultures of resting fibroblasts

Factor	Cells	Reference
FGF	3T3	Gospodarowicz, 1974 [5]
EGF	3T3	Armelin, 1973 [6]
Somatomedin B	Fibroblasts of various sources	Hall et al, 1975 [7]
Somatomedin C	Chick embryo fibroblasts	Van Wyk et al, 1975 [8]
NSILA	Chick embryo fibroblasts	Morell & Froesch, 1973 [9]; Smith & Temin, 1974 [10]
S1 and S2	Rat embryonic fibroblasts	Hoffmann et al, 1973 [11]
Fibroblast mitogen	WI-38, GWF (human fibroblasts)	Houck & Cheng, 1973 [12]
Serum factors	Human lung fibroblasts	Michl & Rezacova, 1966 [13]
	3T3	Todaro et al, 1967 [14]; Holley & Kiernan, 1968 [15]; Paul et al, 1971 [16]
	Mouse embryonic fibroblasts	Healy & Parker, 1970 [17]
Insulin	Chick embryo fibroblasts	Temin, 1967 [18]
	3T3	Yarnell & Schnebli, 1974 [19]; Holley & Kiernan, 1974 [20]
Blood platelets	3T3	Kohler & Lipton, 1974 [21]; Antoniades et al, 1975 [22]
Growth factor from cartilage	3T3	Klagsburn et al, 1977 [23]
MSA	Chick embryo fibroblats	Pierson & Temin, 1972 [24]
Factors released by cells	BHK	Shodell, 1972 [25]
	Chick embryo fibroblasts	Dulak & Temin, 1973 [26]
	3T3	Smith & Temin, 1974 [10] Bürk, 1976 [27]
Thrombin	Chick embryo fibroblasts	Lan Bo Chen & Buchanan, 1975 [28]
Proteolytic enzymes	3T3	Burger, 1970 [29]
	Chick embryo fibroblasts	Sefton & Rubin, 1970 [3]
Prostaglandin $F_{2\alpha}$	Rat embryonic fibroblasts	Frank et al, 1973 [31]
	3T3	Jimenez de Asua et al, 1975 [32]
Putrescine	Human fibroblasts	Pohjanpelto & Raina, 1972 [33]
Glucocorticoids	3T3	Thrash & Cunningham, 1973 [34]
	Human fibroblasts	Cunningham et al, 1974 [35]
cGMP	3T3	Seifert & Rudland, 1974 [36]
PMA	3T3	Sivak, 1972 [37]

Abbreviations: NSILA = nonsuppressible insulin-like activity; GWF = human cutaneous fibroblasts; MSA = Multiplication stimulating activity; PMA = phorbol myristate acetate.

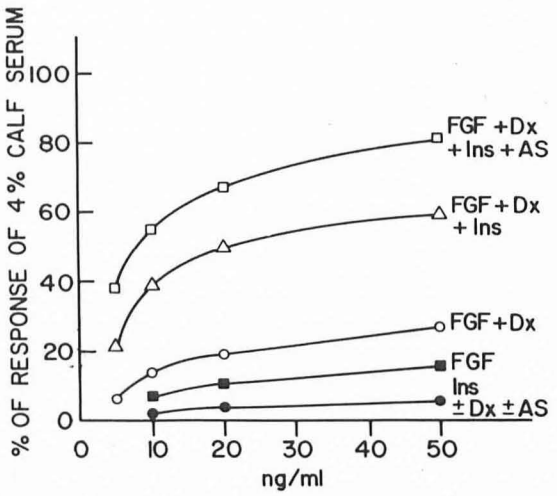

FIG 3. Initiation of DNA synthesis by known factors. The figure gives the percentage replacement of 4% calf serum achieved by: ●, insulin, either alone or with added dexamethasone (*Dx*) and/or heated ammonium sulfate fraction (*AS*) of mouse serum; ■, varying concentrations of FGF alone; ○, varying concentrations of FGF plus 0.4 μg/ml of dexamethasone; △, FGF plus 50 ng/ml of insulin plus 0.4 μg/ml of dexamethasone; □, FGF plus 50 ng/ml of insulin plus 0.4 μg/ml of dexamethasone plus 0.2 mg/ml of protein from the heated (10 min at 100°C) 50 to 70% ammonium sulfate fraction of mouse serum.

of DNA synthesis in quiescent cultures (3.7×10^{-10} M). Only about 25% of the binding sites need to be occupied in order for cells whose growth was arrested in G_1 to enter the S phase. After binding of EGF to the cells, it is rapidly internalized and degraded. As the bound EGF is internalized, most of the membrane receptors become inaccessible to added fresh hormone and remain inaccessible for several hours in the continued presence of nanogram quantities of the hormone. Maximal binding is achieved approximately 40 min after addition of the hormone to the medium, and degradation of the hormone bound at that time is nearly complete 2 hr later. However, the initial binding and subsequent degradation of the bound hormone are not sufficient to stimulate DNA synthesis; the hormone must remain in the culture medium for several hours and presumably bind repeatedly to receptors in order to stimulate DNA synthesis. Any explanation of the relationship between binding of the hormone to the cells and the initiation of DNA synthesis has to take into account this time differential between initial binding and initiation [40].

The role of serum or polypeptide hormones such as EGF in density-dependent regulation can be studied experimentally. The saturation density of the fibroblast culture is proportional to the serum or growth factor concentration. The higher the original saturation density, the higher the serum or growth factor concentration must be raised in order to initiate DNA synthesis. The dependence on growth factors can be studied at different cell densities in different dishes and at different densities in the same dish. An example of the latter type of experimental situation is referred to as "wound healing."

"WOUND HEALING"

"Wound healing" experiments provide some of the best examples of "density-dependent regulation." After a culture has become quiescent, a narrow strip of cells is scraped off the dish leaving a wound in the monolayer. The medium and concentration of serum factors remain unperturbed. Cells at the edge of the wound then migrate into it and divide (Fig 4). Growth stops when the wound reaches the same cell density as the surrounding monolayer. The cells in the surrounding layer remain quiescent in the same medium in which the cells at the wound edge and within the wound divide.

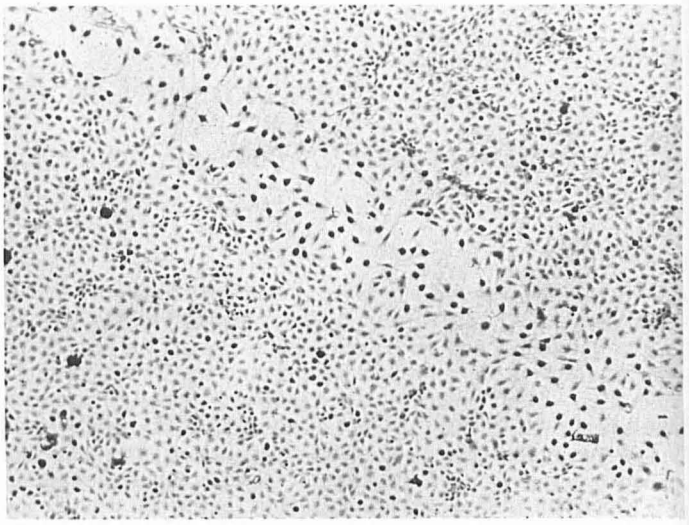

FIG 4. Autoradiography of a "wound healing" experiment with 3T3 cells. Twenty-four hours after "wounding" of the monolayer, ^{3}H-thymidine was added for a period of 24 hr; the cells were then prepared for autoradiography. The autoradiogramm was kindly provided by Dr. Renato Dulbecco, The Salk Institute.

The process of wound healing requires serum factors [41,42]. Migration factors that enable the cells to move into the wound in the absence of serum have been isolated, but the cells do not initiate DNA synthesis without the addition of serum or of other macromolecular-weight growth factors from serum [42].

To initiate the cells in the monolayer surrounding the wound, one must increase the serum concentration or growth factor concentration in the medium. This greater serum requirement for the crowded cells in the monolayer is not fully understood, but it is known that polypeptide growth factors are neutralized or destroyed by cells [4]. As the cell density increases, the rate of destruction probably also increases, a phenomenon that may explain the higher serum requirement of crowded cells since destruction of the serum factors would lead to the existence of a diffusion boundary layer for the growth factors [43]. In addition, it is likely that the surface area and mobility of cells decrease as the cells become more crowded, and this crowding may cause them to be less responsive to a given concentration of growth factors. There is evidence that the surface area of cells is important in density-dependent regulation of growth [44–46].

EFFECT OF NUTRIENTS

Manipulations of the culture medium have demonstrated that low concentrations of low-molecular-weight nutrients such as amino acids, glucose, or ions in the presence of normal concentrations of serum can lead to G-1 arrest of the cell culture. One can then stimulate the cells to initiate growth and to proceed through the cell cycle by raising the concentration of the nutrients [47,48]. Dulbecco and Elkington were able to stimulate arrested Balb/C 3T3 cells by increasing the Ca^{++} concentration above the normal level [49]. These results indicate that the growth of mammalian cells can be regulated by changes in the concentration of nutrients inside the cell. The function of high-molecular-weight growth factors in serum could be to increase the concentration of one or more nutrients inside the cell by changing the uptake of nutrients through interactions with the cell membrane. Alternatively, serum factors and nutrient concentrations may operate independently on a common regulatory mechanism.

EARLY EVENTS AFTER STIMULATION

Investigations into the stimulation of quiescent cultures have revealed a number of cellular changes occurring in response to

the addition of growth factors. Herschko et al [50] suggested the term "pleiotypic response" for these collective changes. The changes include the increases in the transport of nutrients and ions [48,50–53], changes in RNA and protein synthesis [50, 54,55], changes in the intracellular concentrations of cyclic nucleotides [35,56,57], and the assembly of microtubules [58].

One of the earliest changes detected after growth stimulation is a decrease in the membrane potential as observed by Hülser and Frank [59]. The membrane potential between the inside and outside of a mammalian cell mainly depends on differences in the intra- and extracellular concentrations of potassium ions as well as on the permeability of the plasma membrane to these ions. The Na^+- K^+-activated ATPase maintains this membrane potential. The membrane potential of rat embryo fibroblasts was reported to decrease from −50 mv to a minimum of −17 mv within 3 min after exposure to serum growth factors; after 2 hr of incubation in the presence of growth factors, the membrane potential was restored to its original value. Depolarization was not observed when inactive serum fractions were added to the cultures. There also was no change in the membrane potential of a transformed rat cell line that grew independently of serum [59].

Phosphatidyl inositol turnover is increased during the first few minutes after fibroblasts arrested during G_1 have been stimulated by growth factors.* The specific activity of ^{32}P-phosphate in phosphatidyl inositol increases seven- to eightfold 30 min after the addition of serum or growth factors, whereas the other phospholipids show no or only a slight increase in synthesis during this time. When rat embryo fibroblasts are prelabeled with ^3H-inositol, up to 15 to 20% of the total radioactivity is eliminated from the phospholipid fraction 20 to 40 min after stimulation with serum. Within 1 to 2 hr the original value is restored.

Among the early events, that receiving the most attention has been the changes in the intracellular concentration of cyclic nucleotides. Many investigators have reported a decrease in cAMP when resting fibroblasts are stimulated to grow [56, 60, 61]. Decreased levels of cAMP have been reported for logarithmically growing cells and for transformed cells; resting cells reportedly have high concentrations [61,62]. In addition, dibutyryl-cAMP added to growing fibroblasts can reversibly inhibit DNA synthesis and growth [63], as well as the uptake of uridine, leucine, and 2-deoxyglucose [61]. Synthesis of phosphatidyl inositol decreases by 50% in the presence of dibutryl-cAMP without a change in the main cellular phospholipid component, phosphatidylcholine [64]. Kram, Mamont, and Tomkins concluded that cAMP may act as a regulator of the "pleiotypic response" [61].

Reports from studies made with cultured lymphocytes [57] showed that although cAMP levels were unaltered after growth stimulation, the concentration of another cyclic nucleotide, cGMP, increased. It was suggested in 1973 that the ratio of cGMP to cAMP is important for the commitment of cells to the initiation of DNA synthesis. This model became popularly known as the "Yin-Yang" hypothesis [65]. Voorhees and coworkers observed in rapidly dividing epidermis, e.g., psoriatic lesions, that the concentration of cAMP is decreased and that of cGMP is increased [66]. Shortly thereafter, Seifert and Rudland also observed an increase in cGMP and a decrease in cAMP after stimulation of fibroblasts [36]. Although all of these results favor the idea of cyclic nucleotides playing a regulatory role in growth control, there are other reports that do not. For example, other investigators have not observed an increase in cGMP in fibroblasts after stimulation [67]. It is also reported that in hepatocytes and lymphocytes, DNA synthesis is preceded by an increase instead of a decrease in cAMP [68,69]. These conflicting observations have created doubts concerning the importance of cyclic nucleotides in growth regulation. In addition, because cyclic nucleotides are involved in many differentiated functions of cells, it seems doubtful that they are the main regulator of cell growth.

The role that the "pleiotypic response" plays in the initiation of DNA synthesis is unclear. Because of the long lag time between addition of growth factors and initiation of DNA synthesis, it is reasonable to assume that a program of events is necessary for initiation. The events described above, along with changes in RNA and protein synthesis, seem to be a generalized response of cells to stimulation by serum or hormones, but these observations tell us little of the mechanism of cellular commitment to DNA synthesis. A recent report describing a positive pleiotypic response in an adrenal cell line under conditions that led to differentiation and inhibition of DNA synthesis [70] makes questionable the causal relationship between these early events and initiation.

CHALONES

A group of compounds, referred to as chalones, are reported to inhibit cell growth. Chalones are cell-specific but not species-specific, and their action is reversible.

Houck, Weil, and Sharma obtained a fibroblast chalone from medium extracts of cultured human fibroblasts and from tissue extracts of fibroblasts [71]. These extracts were active only on fibroblasts and cells from a human osteosarcoma, and not on epidermal, lymphocytic, or other types of cells [72]. The chalone molecules isolated from these extracts are trypsin-labile proteins which form strong complexes with RNA. They have a molecular weight of less than 10,000 but more than 1,000 daltons, are cationic, and contain mannose [73]. Because there appears to be no direct interaction between chalones and fibroblast mitogens, it is suggested that cellular proliferation is controlled by a competition between chalones and growth factors for cellular receptor sites [74].

CONCLUSIONS

We still do not know the mechanism of action of growth factors or, more importantly, the way in which a fibroblast becomes committed to initiate DNA synthesis. It does appear that many growth factors can stimulate fibroblasts and that these growth factors interact and are more active in combination than alone. It is likely that different factors act on different types of cells and that some larger molecules are only the carriers for the active smaller ones that are not separated by the isolation procedure. Some factors may not be specific but may express growth activity through nonspecific interactions with cell surface receptors. Another possibility is that there exists a whole set of factors working together in a "cascade" as in the clotting, complement, or kinin systems.

How a fibroblast chalone would fit into this system is unanswered. Although we do not have much information concerning a growth factor-chalone interrelationship in regulating growth, both may compete for the same receptor site at the cell surface. We probably will have to wait until the molecule has been purified before we can hope to understand its mechanism of action.

The early events inside stimulated cells tell us little about the way in which a cell is committed to the initiation of DNA synthesis. Except for the more general effects of increased RNA and protein synthesis, all the other events appear to be unnecessary for initiation of DNA synthesis. They may be only side effects, such as an expression of lysosomal activation to internalize and digest the foreign substances attached to the cell surface.

The main question behind all these investigations is, "How applicable to the in vivo situation are these data?" There exists a large gap between our observations of tissue culture and

* Ristow H-J, Hoffmann R, Frank W, Paul D: Phospholipids with special emphasis on changes in phosphatidyl inositol that accompany changes in cellular growth state, Symposium on Nutritional Requirements of Vertebrate Cells in Culture, Lake Placid 1975. Edited by P Chapple and RG Ham, to be published.

observations of normal tissues in situ. Results from tissue culture studies give no explanation of why dividing fibroblasts in a wounded area in the organism are first observed after 2 days [75] and why these fibroblasts do not come from the surrounding dermis but rather from walls of adjacent blood vessels or nearby sarcolemma [76]. The idea of a growth factor released in large amounts from the injured blood vessels or of a chalone removed from the tissue does not fit in with this observation. The assumption that cells such as macrophages, which precede fibroblasts into the wound area, may influence migration and division of fibroblasts is probably more compatible with the observed process of wound healing in vivo. It has been reported that substances released from dying macrophages stimulate fibroblast activity, which results in fibrosis [77].

Although experimental work with cultured fibroblasts has helped us to overcome the difficulties and complications found in the intact organism and has provided us with a greatly increased understanding of growth regulation, it will be necessary to find ways to bridge the gap back to the intact organism before we can obtain answers concerning regulation of fibroblast growth.

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Announcement

A meeting, entitled "Progress in Cutaneous Immunopathology," conducted under the auspices of the "Institut National de la Santé et de la Recherche Médicale" will be held October 27–28, 1978, in Lyon, France.

The meeting will be conducted in English and French and will comprise 4 sections: (1) In vivo fixation and cytotoxicity of antibodies on skin diseases, (2) immune complexes and complement in skin disease, (3) surface markers and antigens of the epidermal cells and cutaneous lymphoma cells, and (4) chemotaxis and cutaneous diseases.

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