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DEMONSTRATION AND CHARACTERIZATION OF AN EPIDERMAL ANGIOGENIC FACTOR*

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ABSTRACT

In this study the vasoproliferative effects of adult hamster epidermis and epidermal homogenates on the microvasculature of the hamster cheek pouch were observed.

Implants of epidermis stimulated a rapid growth of new vessels both upon direct implantation and when separated from the vascular bed by a microporous filter. No significant vasoproliferation was seen with dermis, polythene, dialysis membrane, Millipore filter, with epidermis placed on or within sheets of dialysis membrane, or when the epidermis had been inactivated by heat treatment.

Epidermal homogenates, Millipore filtrates, and dialysis tenates induced new vessel growth whereas similar preparations from dermis did not. Activity was found to be present in the aqueous but not the organic phase following ethyl acetate extraction and was deactivated by heating or removed by precipitation with trichloroacetic acid. Vasoproliferative activity was not affected by maintenance at 4° C for up to 7 days.

These observations are interpreted as evidence for a specific, heat-labile, diffusible but nondialyzable protein "epidermal angiogenic factor."

Complex epidermal–dermal interactions have been implicated in the regulation of physiologic processes as diverse as fibrinolysis [1–3], epidermal kinetics [4, 5], and the origin and maintenance of epidermal specificities [6–8]. More recently, such an interplay has been cited as a possible regulatory factor in the control of the cutaneous microvasculature [3, 9] with the suggestion that the epidermis may be capable of acting upon the dermal vasculature, perhaps through the elaboration of a diffusible substance or substances.

Clinical observations reveal wide variation in patterns of cutaneous blood supply with age, disease, or traumatic injury, implying that changes in the epidermis are capable of inducing concomitant alterations of dermal blood vessels. Warren and Shubik [10], Greenblatt and Shubik [11], and Folkman and colleagues [12] have demonstrated an angiogenic factor (or factors) in a variety of human and animal tumors, while Nishioka and Ryan [13] have recently published evidence implying the production of both vasostimulatory and vasoinhibitory factors in neonatal hamster epidermis.

In this study we have investigated the vasoproliferative impact of adult hamster epidermal implants and homogenates on the microvasculature of the hamster cheek pouch. We report the demonstration and characterization of a proposed epidermal angiogenic factor.

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MATERIALS AND METHODS

The Hamster Cheek Pouch Technique

One hundred and seventy-one randomly bred adult male and female Syrian golden hamsters (*Mesocricetus auratus*) of 100 to 160 gm body weight were individually housed in plastic cages and maintained on rat and mouse Breeder diet (Type FFG [M] Dixon, London) and water ad lib. As prophylaxis against infection, tetracycline hydrochloride (Achromycin, Lederle Laboratories) 7 gm/l was added to the drinking water. The animals were anaesthetized with pentobarbitone sodium (Nembutal, Abbot Laboratories) and the hair removed with electric clippers from the area of the left cheek pouch followed by depilation. Two-piece, transparent acrylic cheek-pouch chambers of traditional design [14, 15] were inserted into the left cheek pouch according to the technique of Warren and Shubik [10], Greenblatt and Shubik [11], and Greenblatt, Choudari, Sanders, and Shubik [14].

All microscopic observations were made and photographically recorded on the Leitz Orthoplan microscope with special long-working-distance Ultropak objectives. Light was directed from a high-intensity external source through a perspex light-pipe placed in the cheek pouch, and hence upward through the transparent chamber into the Leitz ocular system.

The Experimental Design

Because of their immunologically "privileged" status [15, 16], cheek-pouch preparations are able to receive implanted materials with relative equanimity. We utilized this unusual but useful quality of immunologic quiescence to study the angiogenic stimulus of three separate but related series of epidermal components and controls.

First, 4.0-mm-diam punch biopsies of clipped and depilated skin from donor adult hamsters were incubated

at 4° C for 2 hr in solutions of 2 M KBr to separate epidermis from dermis, good separation at the dermoepidermal junction being verified under the dissecting microscope. The 4.0-mm discs of tissue so obtained were then implanted directly, or following further treatment, onto the vascular bed of the hamster cheek pouch as follows: (a) epidermis (in "normal" and inverted orientation), (b) heat-treated epidermis (80° C × 30 min), (c) dermis.

To eliminate the possibility that the vascular changes seen following implant of skin components were due to nonspecific effects of an inflammatory nature, "inert" materials—polythene, cellophane dialysis membrane (Visking), or microporous cellulose filter (Millipore, type TH, 25 μm thick, 0.45 μm pore size; Millipore Ltd.)—were subjected to the same preparative procedures as the skin and then implanted onto cheek-pouch membrane.

Second, in transfilter diffusion studies, epidermal implants, 4 mm in diam and obtained as above, were separated from the cheek-pouch wall by 6.0-mm discs of Millipore filter (25 μm thick, pore size 0.45 μm) or dialysis membrane. In further efforts to preclude direct cellular contact with the vascular bed, 4.0-mm epidermal implants were placed within carefully folded sheets of both Millipore filter and dialysis membrane.

Next, 4 cm × 3 cm sheets of hamster epidermis, split from donor hamster skin by 2 M KBr, as above, were washed in sterile Eagle's Minimum Essential Medium (Eagle's MEM, Wellcome Reagents, Ltd., England) and homogenized (Ultraturrax TP ION, Scientific Instruments Centre Ltd., London) in sterile physiologic saline in the cold for 5 min. Portions of this crude homogenate were centrifuged at 105,000 × *g* for 1 hr (MSE superspeed 50 T.C. refrigerated centrifuge; rotor 2410; 0–7° C) to produce a supernatant fraction free of cellular debris; the lysosomal, microsomal, and mitochondrial subcellular fractions were discarded (Table I). The supernatant was then passed through a Millipore filter (Type TH, 125 μm thick, pore size 0.22 μm) and part of the resulting filtrate dialyzed in the cold against first tap water and, subsequently, physiologic saline. For control purposes similar preparations were also made from hamster whole skin and from dermis. Finally, to eliminate the possibility of an inflammatory reaction in the pouch vessels due to bacterial contamination, all solutions were sterilized by passage through a sterile bacteriologic filter immediately prior to application to the cheek-pouch membrane. Each of these preparations (homogenates, filtrates, dialysis tenates) was then studied by two methods: first, direct application to the vascular bed; second, saturation and implantation of 4.0-mm discs of Millipore filter.

Characterization Studies

To further characterize the angiogenic potential of epidermal filtrates and dialyzed material, these preparations were subjected to the following treatments prior to implantation: (a) heating at 56° C and 80 ° C for 1 hr and ½ hr, respectively, (b) protein precipitation with trichloroacetic acid (0.1 ml 10% TCA/2.0 ml epidermal filtrate), followed by sequential dialysis in the cold against tap water and physiologic saline, (c) ethyl acetate extraction, and (d) storage at 4° C for 7 days.

Fibrin Studies

A further set of experiments was designed to investigate the possible role of fibrin deposition in epidermally induced vasoproliferation. First, histologic sections of pouch membrane bearing Millipore discs saturated with

TABLE I

Preparation of epidermal fractions

1. Separated epidermis* (2M KBr, for 2 hr at room temp and washed in Eagle's medium × 3)

↓ homogenized in cold in 3 ml sterile physiologic saline

2. Epidermal homogenate*

↓ centrifuged 105,000 × *g* for 1 hr in the cold; supernate filtered (Millipore, 0.22 μm pore size)

3. Cell-free epidermal filtrate*

- dialyzed in cold → 4. Epidermal dialysis tenate*
- 56°C, 1 hr / 80°C, ½ hr → 5. Heat-inactivated filtrate*
- 4°C, 7 days → 6. Cold-stored filtrate*
- TCA addition → 7. Protein-precipitated filtrate*

ethyl acetate extraction

- aqueous phase → 8. Aqueous extract*
- organic phase (dried, reconstituted in sterile saline. → 9. Organic extract*

* Each of these preparations was bioassayed for angiogenic activity on the hamster cheek pouch.

epidermal filtrate were stained with H & E; Mallory's phosphotungstic acid hematoxylin (PTAH), and fluorescein isothiocyanate-labelled rabbit antihuman fibrinogen (RaHu/FBG/FITC, NORDIC batch 1170, F/P: 4/14, dilution 1:20). Second, discs of Millipore filter were dipped immediately into fresh mixtures of lyophilized human fibrinogen (1.0 ml of 0.5% solution, Kabi, Stockholm) and bovine thrombin (2 N.I.H. units, Parke Davis). The discs were placed in moist Petri dishes for 30 min for fibrin clot formation and then implanted into hamster cheek-pouch chambers; these implants were compared with control discs saturated with physiologic saline and Eagle's Medium. Also, epidermal implants and Millipore discs saturated as before with epidermal filtrate were placed in a solution of plasmin (100 μg/ml, Koch-Light Laboratories Ltd.) for 30 min at room temperature and implanted onto the cheek-pouch bed; the effects of these discs were contrasted with those of similar discs saturated with epidermal filtrate alone and others saturated with Eagle's Medium and plasmin.

The specificity of the technique for epidermal/dermal separation was monitored by histologic study of stained preparations of epidermis and dermis. Separation was found to occur at the dermoepidermal junction with a minimum of cross contamination of one tissue with the other. Histology was also used to verify the viability of the implanted tissues after various implant times; viability was assessed by comparison with the histologic structure of freshly prepared skin.

RESULTS

Following insertion of chambers (without implants) into the cheek pouch a reduction in the rate of blood flow, particularly through venules, was observed, together with a moderate leukocytic infiltrate. This latter caused a loss in transparency of the membrane and microscopic definition was impaired. However, these responses to chamber insertion had resolved by day 3 or 4 and thereafter blood flow appeared normal and the membrane suitable for photomicrography by transillumination (Fig. 1).

Direct Implant Studies

The presence of implanted epidermis on the stroma of the pouch wall caused a marked departure from this normal vascular pattern in the immediate vicinity of the implant. The changes occurring in response to the presence of the epidermal graft and confined to vessels located up to 1–2 mm of the edge of the implant, could be categorized in two main groups: alterations in preexisting vessels; induction and potentiation of new vessels.

Changes falling into the first category appeared early, frequently in the first 24 hr postimplant, and included massive tortuosity particularly of small veins and venules; engorgement and saccular dilatation (giving the appearance of "beading" and "sausaging") occurred in the patent capillaries whilst many others, previously closed, had opened up and the general impression was of a much more highly vascularized region (Fig. 2).

These early vascular changes were followed by a

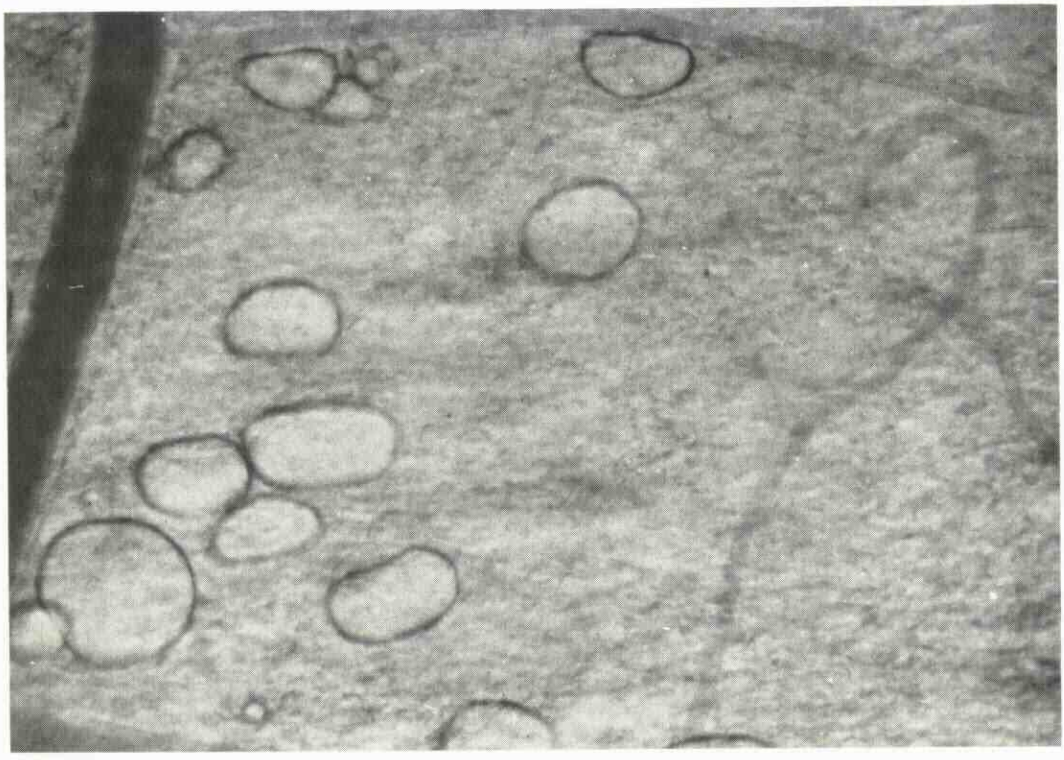

FIG. 1: Vascular architecture of normal cheek pouch, 3 days after insertion of chamber. Note relative paucity of patent capillaries. × 110.

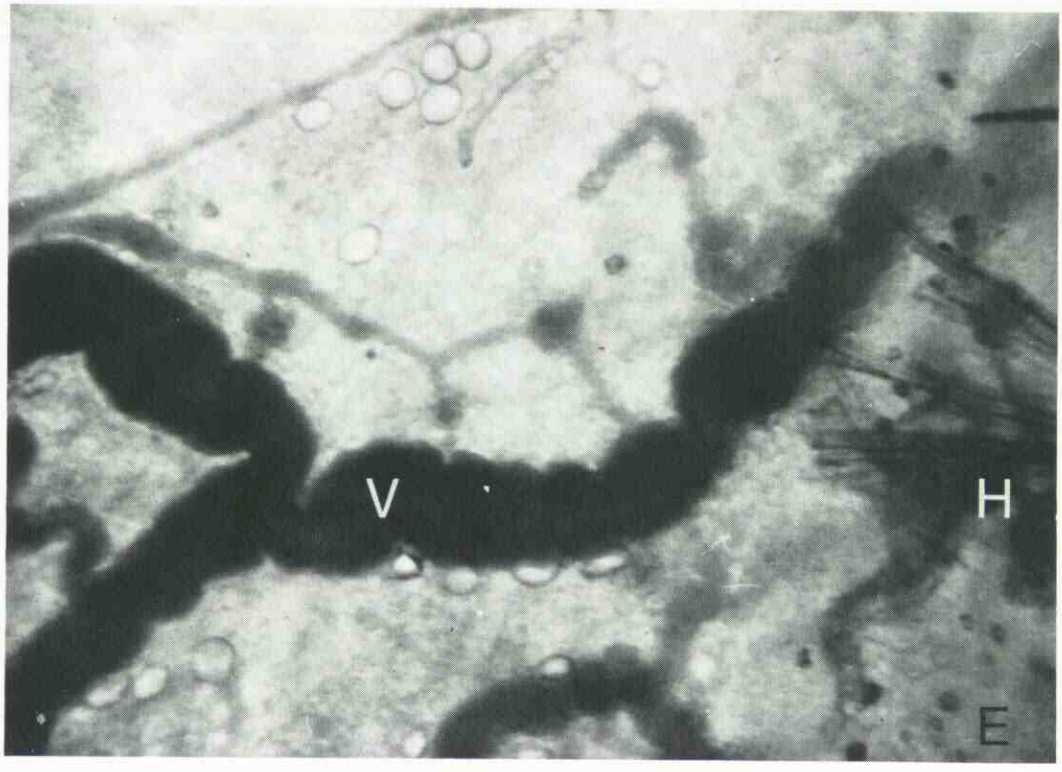

FIG. 2: Early effects of epidermal implants on the vascular bed. Marked saccular dilatation and tortuosity of vessels (V) adjacent to the implant (E) at day 3 postimplant. H: hair tuft in epidermal implant. × 110.

phase of new vessel induction. Commencing at around day 3 or 4 postimplant; saccular capillary sprouts arose from the tortuous venules and grew towards the edge of the implant. By day 5 or 6 postimplant small loops and blind-ended capillary sprouts containing static erythrocytes were seen to reach over the edge of the implant (Fig. 3). These vessels continued to grow up and over the epidermal implants, reaching a maximum growth (up to 70% of the implant radius) between day 6 and day 8. By this stage the linear growth pattern was superceded by complex arborization of anastomozing new vessels and a vigorous circulation overlying the implant was established. The sequential appearance of these features at the appropriate time after implant was taken as the criterion for a positive vasoproliferative effect (Table II).

On no occasion were vessels seen to overgrow the epidermal implant completely or to link with vessels growing in from the opposite edge. Minute foci of hemorrhage appeared after 12–14 days and microscopic resolution deteriorated to such an extent that observations were terminated at this time.

The progression of vascular change outlined in Table II was seen in all of 15 experiments where small pieces of adult hamster epidermis were applied to the cheek-pouch membrane. No differences, either in quality or intensity of response, could be seen in 5 chambers where the epidermis was applied in "inverted" (with the stratum corneum in contact with the membrane), as opposed to normal, orientation.

On the other hand, 5 epidermal grafts which, prior to insertion of the chamber, were heated in a water bath at 80° C for 30 min, or 56° C for 1 hr, failed to induce proliferation. Blood flow in one or two of the chambers increased slightly in the vicinity of the heat-killed grafts but for up to 8 to 10 days the vascular network remained essentially normal. In 4 of the 5 chambers containing these heated epidermal implants, an area of necrosis appeared around the graft, commencing about day 8 to 10, and rapidly spread outwards. On removal of the chamber the pouch wall underlying the implant was found to be completely eroded leaving a small circular hole surrounded by a compact plexus of small blood vessels. This effect was attributed to the release of proteolytic enzymes from the autolyzing graft.

Eight implants of dermis, 6 of polythene film, and 6 of cellophane dialysis membrane, after being subjected to similar preparative procedures as the epidermal implants, produced no visible changes in the topography of the vascular bed. On the other hand, implants of Millipore filter membrane did occasionally cause some new vessel growth but this effect was generally restricted to implants of the thick membrane (125 μm) and did not occur until comparatively late (10–14 days after implant). The

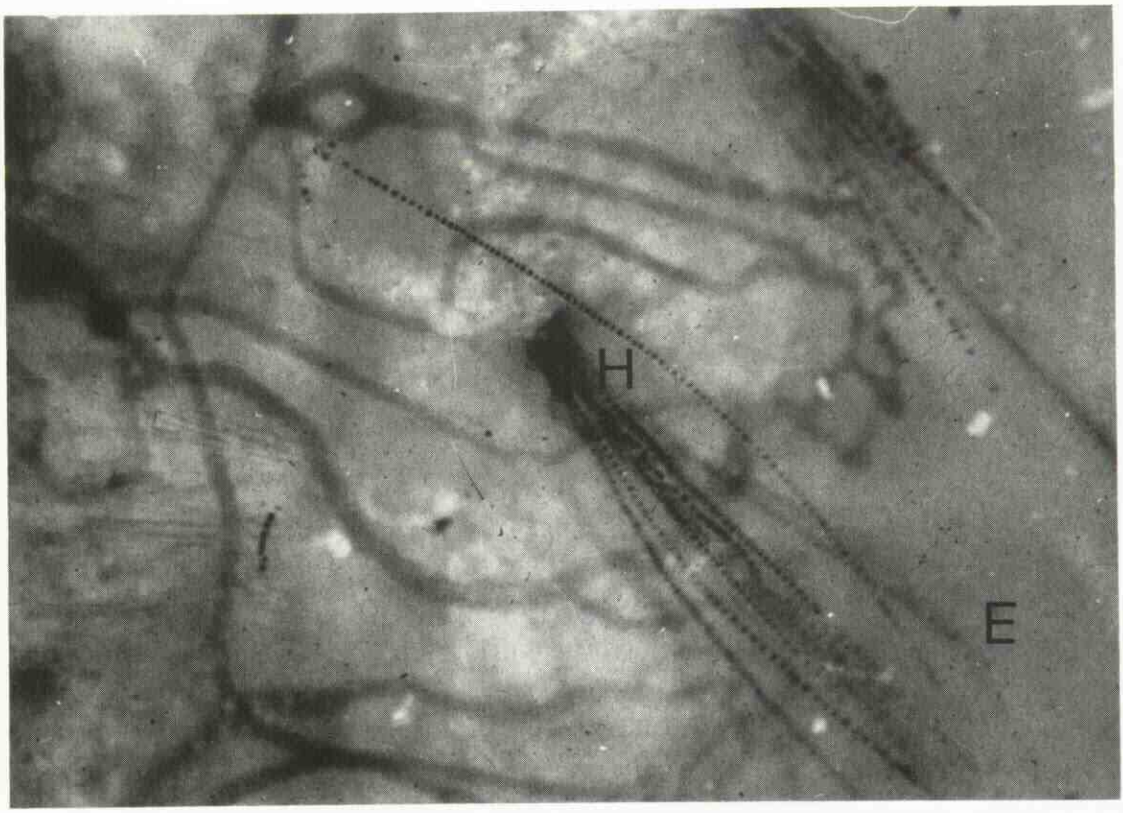

FIG. 3: Vascular overgrowth of implant. New vessels growing up and over the rim of the epidermal implant (E) and anastomozing to form a new vessel network at day 6 postimplant. Note actively growing hair tuft (H) in the implant. × 110.

TABLE II

Outline of the sequential changes occurring in response to a positive angiogenic stimulus.

Time after implant	Vascular alterations
24–48 hr	Leukocytic infiltrate
2–3 days	Tortuosity, beading, and sausaging of existing vessels; increased blood flow; increased proportion of patent capillaries
3–5 days	Formation of new vascular sprouts, directionally orientated towards implant
4–7 days	Initial overgrowth of implant edge by blind-ended sprouts containing static blood
6–8 days	Arborization of capillaries; more extensive overgrowth; closed capillary loops with circulating blood only at the edge of implant
7–10 days	Anastomosis of new vessels giving very complex vasculature with brisk circulation

25 μm membranes, when implanted alone, caused little or no vascular change and were considered sufficiently "inert" to be used as the support in transfilter diffusion studies.

Indirect Implant Studies

To further investigate the nature of the angiogenic stimulus present in separated epidermis, 4-mm-diam implants were isolated from the vascular membrane of the cheek pouch by discs of Millipore filter (6 mm diam, 25 μm thick, pore size 0.45 μm). This combination stimulated a vigorous vasoproliferation and eventual overgrowth of both the filter and epidermal fragment comparable in intensity and timing to epidermis alone (Fig. 4). An intense proliferation of small vessels both beneath and over the implant was confirmed histopathologically (Fig. 5).

When the epidermal preparation was folded within the Millipore membrane as opposed to simply resting upon it, the sequence of vascular reactions was comparable both in intensity and timing, showing tortuosity and "beading" in the first few postoperative days followed by vascular outgrowth beneath and at all edges of the filter.

On the other hand, in 6 chambers where cellulose dialysis membrane was substituted for the Millipore barrier the reactions were restricted to vessels beyond the edge of the membrane, being most intense in the sector where the epidermal fragment (which had been deliberately placed off-center) was closest to the edge of the barrier disc. Similarly, the response to epidermis placed in

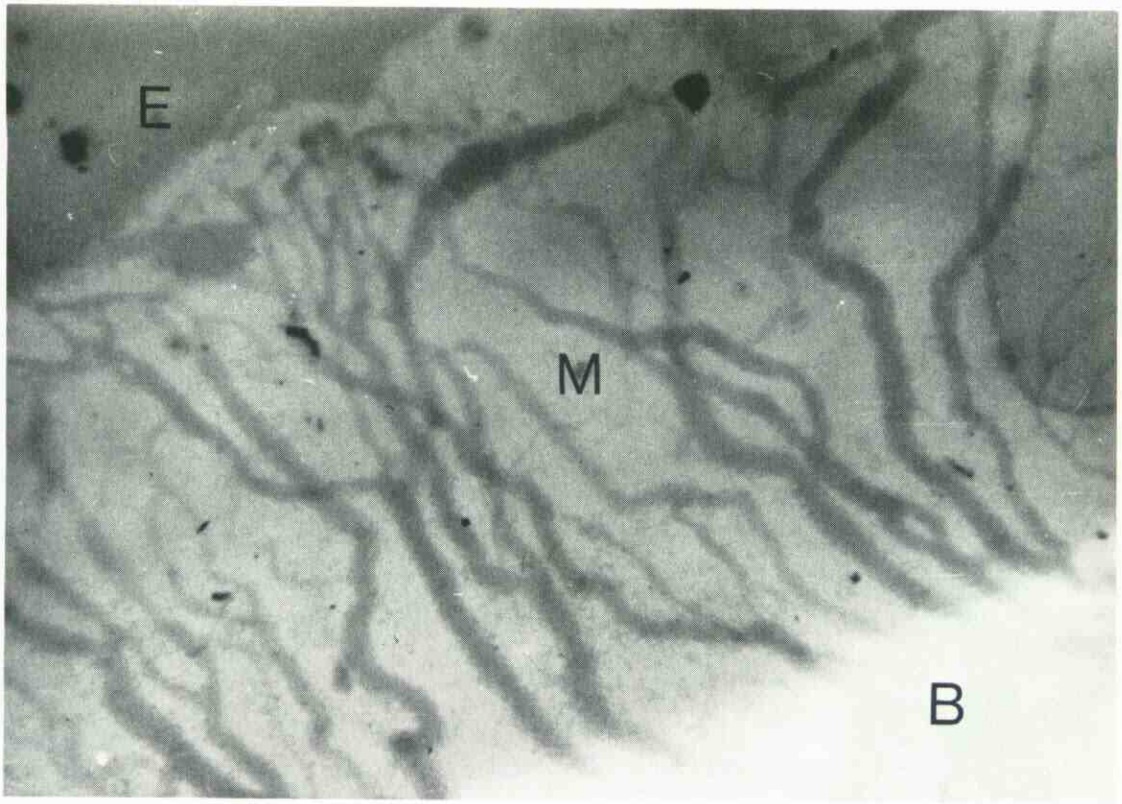

FIG. 4: Vascular overgrowth. Epidermal implant (E) separated from the vascular bed (B) by a disc of Millipore filter (M). New vessels arise from the pouch wall to overgrow both the Millipore filter and ultimately the epidermal implant. Day 6. × 110.

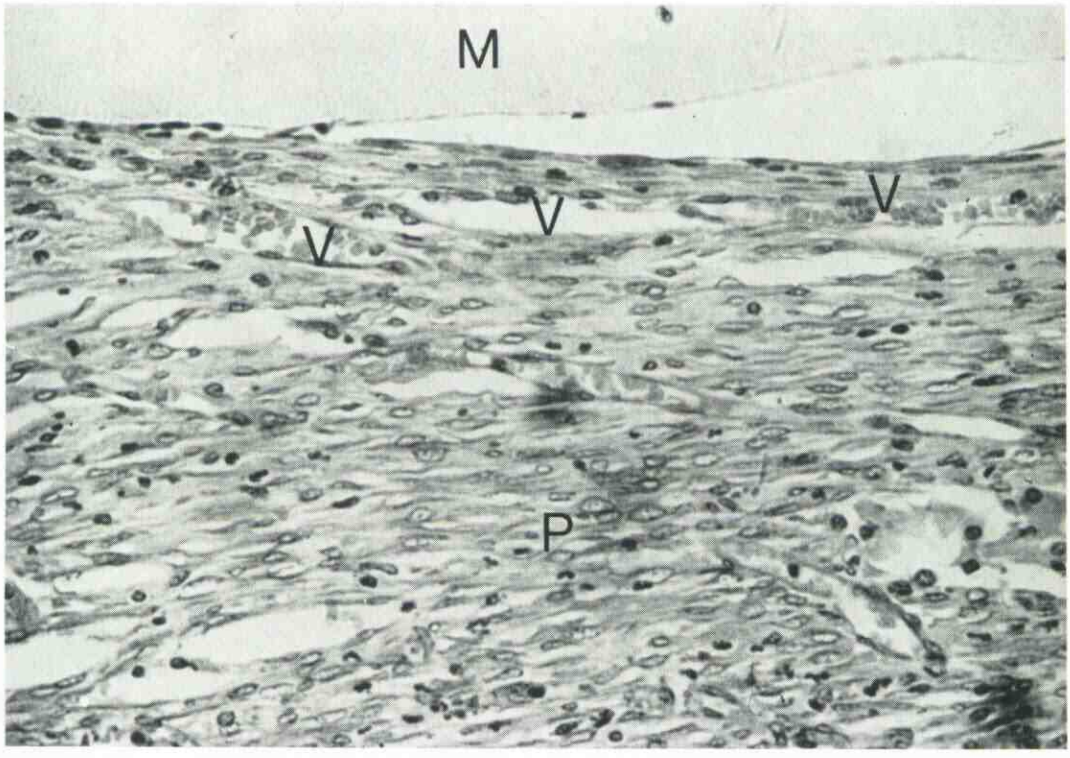

FIG. 5: Histology of pouch wall bearing 6-day implant of epidermis on Millipore filter. Marked dilatation and proliferation of vessels (V) suggesting transfilter diffusion of angiogenic factor. P: cheek-pouch membrane; M: Millipore filter. H & E. × 160.

TABLE III

Summary of the angiogenic capacity of skin tissues and controls

	Implants inducing significant angiogenesis	Implants showing no angiogenic stimulus
Intact Tissues	Epidermis ("normal" orientation) (15)*	Dermis (8)
	Epidermis (inverted orientation) (5)	Heat-inactivated epidermis (80° C for 30 min) (5)
	Epidermis implanted on discs of Millipore filter (6)	Epidermis implanted on discs of dialysis membrane (6)
	Epidermis enclosed within folded Millipore filters (6)	Epidermis enclosed within folded dialysis membrane (6)
Homogenized and Fractionated Tissues	Whole skin homogenate (6)	
	Whole skin filtrate (6)	
	Whole skin dialysis tenate (6)	
	Epidermal homogenate (6)	Dermal homogenate (6)
	Epidermal filtrate (6)	Dermal filtrate (6)
	Epidermal dialysis tenate (6)	Dermal dialysis tenate (6)
	Epidermal filtrate held at 4° C for 7 days (4)	Epidermal filtrate heated (80° C for 30 min; 56° C for 1 h) (4)
		Epidermal filtrate after precipitation with TCA (4)
	Epidermal filtrate: aqueous phase of ethyl acetate extraction (4)	Epidermal filtrate: organic phase of ethyl acetate extraction (4)
		Epidermal filtrate combined with plasmin (supported on disc of Millipore filter) (4)
Noncutaneous Control Implants	Fibrin clot (supported on disc of Millipore filter (6)	Physiologically normal saline (3)
		Eagle's medium (3)
		Dialysis membrane (6)
		Polythene (6)
		Millipore filter (12)

* Numbers of hamsters in parentheses.

a fold of dialysis membrane was apparent only at the open edges.

Epidermal Extracts

Extracts from sheets of separated epidermis were prepared and were tested for angiogenic activity at various stages of purification (Table III). Separated dermis was extracted in an identical manner, the fractions serving as a control of the preparative procedure.

Initially, 0.1 ml aliquots were applied directly to the cheek-pouch membrane but it was found that these spread extensively and produced changes affecting the whole of the observed area. Subsequently, in order to localize the active material and to allow a more prolonged action, 4-mm discs of Millipore filter were saturated with the extracts and layered onto the cheek-pouch wall.

Crude homogenates of epidermis (or of whole skin) applied in this way caused massive tortuosity and a rapid vasoproliferation by the third postoperative day (Fig. 6) which had progressed to overgrowth of the disc by day 5 (Fig. 7).

Dermal homogenates produced no overgrowth and only a mild degree of tortuosity.

Characterization Studies

The vasoproliferative activity was not reduced by Millipore filtration of the crude homogenate nor was it lost following dialysis overnight at 4° C against tap water followed by sterile physiologic saline. The epidermally derived vasoproliferative potential was retained in homogenates held at 4° C for 7 days but was destroyed by heating at 56° C for 1 hr or at 80° C for 30 min (as was the activity of intact epidermis). Protein precipitation with trichloroacetic acid of crude epidermal homogenate or the filtered and dialyzed derivatives of this was accompanied by a loss of the angiogenic principle. Following ethyl acetate extraction of filtered homogenate, the angiogenic factor was retained in the aqueous phase (Table III).

Fibrin Studies

Low-power stereomicroscopy of chambers showing marked vascular proliferation, revealed a distinct layer of translucent gelatinous material overlying the grafts, newly formed vessels appearing to be embedded in this gel. Conventional histology indicated a thin layer of amorphous tissue with a mixed cellular infiltrate (mainly of fibroblasts and histiocytes) extending from the vascular bed to envelop the implanted tissue. Patent blood vessels were clearly demonstrable in this scaffolding (Fig. 8). Histochemical staining with Mallory's phosphotungstic acid [17] showed the characteristic dark blue suggestive of fibrin. Direct immuno-

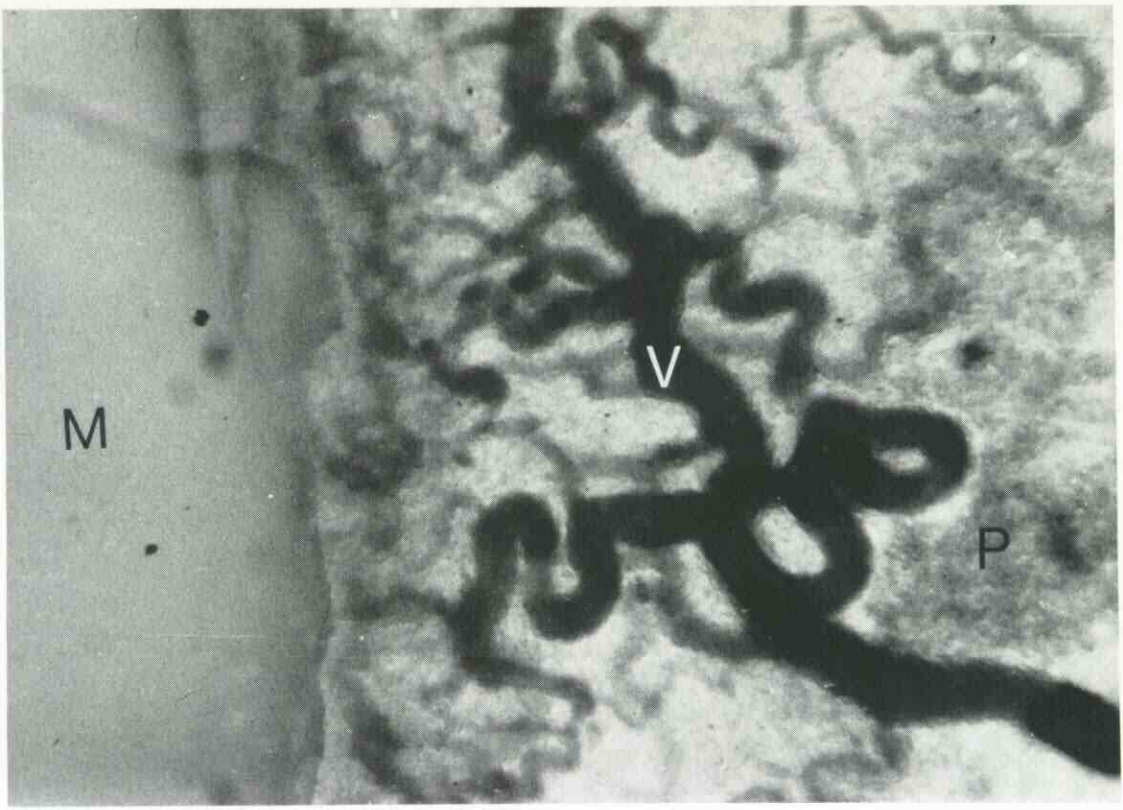

FIG. 6: Effect of Millipore disc (M) saturated with epidermal filtrate. Extreme tortuosity of adjacent vessels (V) by day 3 postimplant. P: cheek-pouch membrane. × 110.

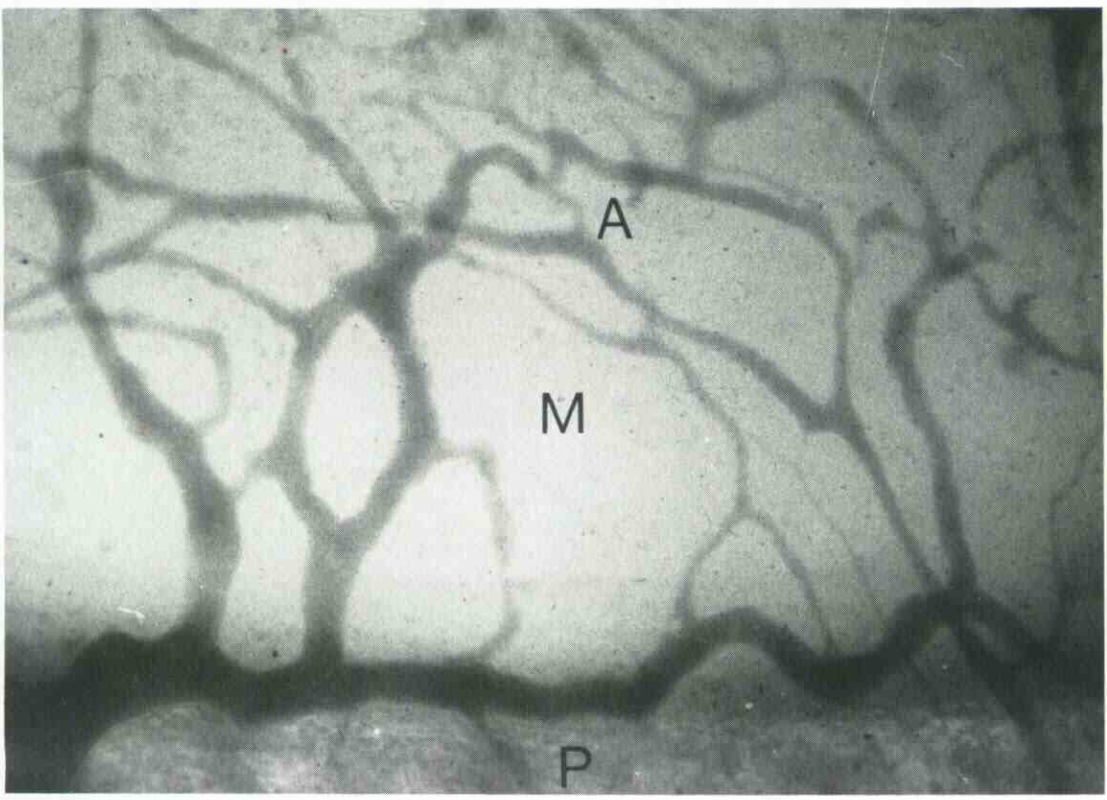

FIG. 7: Effect of Millipore disc (M) saturated with epidermal filtrate: anastomosis. Subsequently the branches anastomoze (A) to complete the circuit and a very rapid blood flow is established. P: cheek-pouch membrane. × 110.

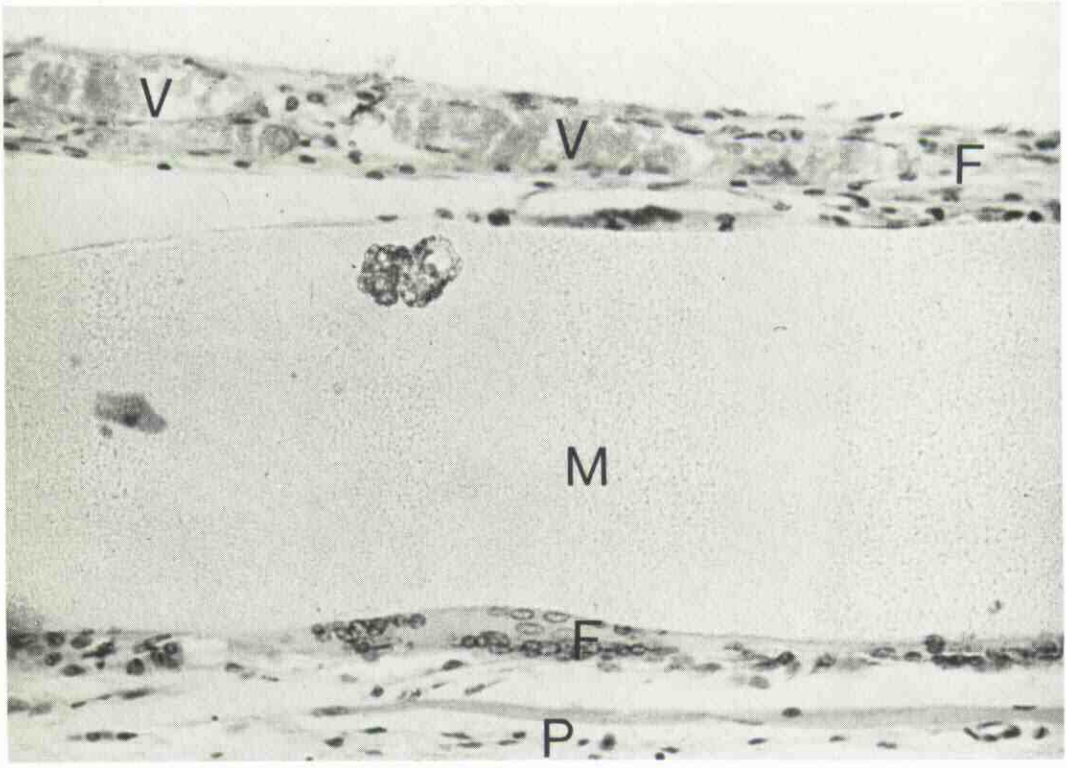

FIG. 8: Histology of the vascular overgrowth. The Millipore filter (M) is overlaid by a thin amorphous tissue (F) with a mixed cellular infiltrate and patent blood vessels (V). Some infiltrate is also apparent between the pouch wall (P) and the filter. Day 6 postimplant. × 250.

fluorescent staining with fluorescein-labelled antifibrinogen antibody (Fig. 9) demonstrated positive apple-green fluorescence both above and below Millipore discs saturated with filtered epidermal homogenate and showing marked vascular overgrowth; control sections incubated in plasmin (100 μg/ml for 30 min at room temperature) prior to staining showed little or no fluorescence. Other sections showed positive fluorescence for complement but were negative for albumin and mixed globulin.

Fibrin-impregnated Millipore discs stimulated extensive vascular overgrowth by day 5–7 whereas similar discs saturated with saline showed no vascular response. The intense vasoproliferative potential of epidermal grafts was suppressed when these were incubated in a plasmin solution prior to insertion in the chamber. Some bizarre beading and tortuosity of vessels in the more remote quadrants of the chamber were noted but none immediately adjacent to the disc itself. It was felt that this effect may have represented activity of fibrin degradation products, but on the other hand a direct action of plasmin on the vascular bed cannot be ruled out.

DISCUSSION

The insertion of a transparent plastic chamber into the hamster cheek pouch provides an excellent technique for the in vivo visualization of small blood vessels in an anatomically and physiologically "normal" setting. Since the cheek pouch is immunologically tolerant, implanted materials may be easily observed for several weeks.

It has been suggested [18] that a fundamental attribute of tumor tissue is its ability to stimulate the formation of new vessels. The survival of an experimental tumor graft depends in large measure on the formation of a new tumor stroma by the host to replace that of the transplant which is absorbed within 48–72 hr [19]. The rapid establishment of a new blood supply to meet the nutritional requirements of the implant is thus of vital importance.

Much evidence in support of the concept of a diffusible vasoproliferative factor from neoplastic tissue has accrued. Warren and Shubik [10] demonstrated the presence of such a vasoinductive component in a Fortner melanoma and subsequently Greenblatt and Shubik [11] were able to show that this factor was capable of passing through a microporous filter membrane. They suggest that this is good evidence for the participation of a humoral factor in tumor angiogenesis although the possibility of cell membrane contact could not be entirely ruled out since electron microscopy of a choriocarcinoma, separated from the hamster cheek-pouch stroma by Millipore membrane, revealed particulate material within the filter pores which may have derived from the

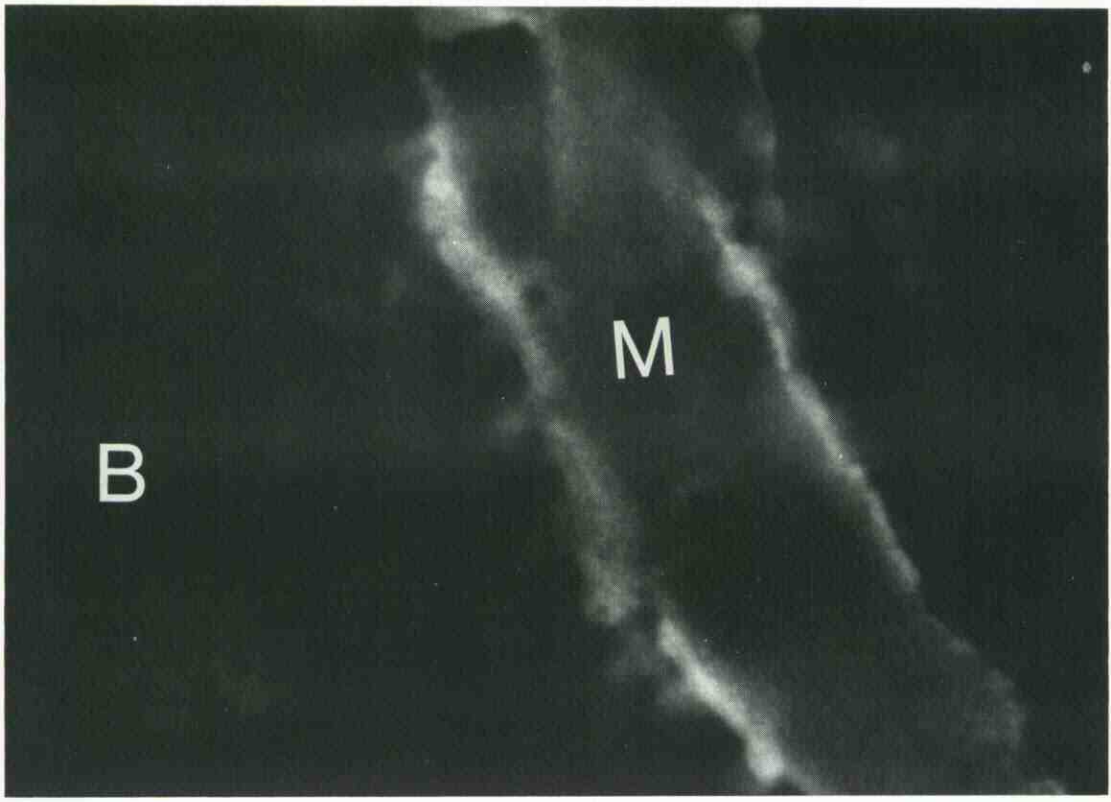

FIG. 9: Fibrin immunofluorescence. Bright bands of fluorescing material present above and below Millipore disc (M) saturated with epidermal filtrate. B: vascular bed. Incubated with antifibrinogen/FITC; day 6. × 110.

implant [20]. Further, Greenblatt [21] was able to demonstrate the presence of cytoplasmic processes from tumor cells within the pores of the supporting filter membrane. In the present study, epidermal homogenates, freed from subcellular particulate contamination by ultracentrifugation, retained angiogenic potential—strong evidence that the epidermis exerts its influence on the blood vessels via a diffusible pharmacologic factor.

Folkman and his colleagues [12] have shown that certain solid tumors produce a factor which is mitogenic for endothelial cells and which they termed the "tumor angiogenesis factor (TAF)." This factor has been isolated from an ascites tumor, Walker 265 sarcoma, from a mouse melanoma, and from a human neuroblastoma.

In general, "normal" tissues do not induce neovascularization when implanted onto the hamster cheek pouch; hamster connective tissue [11], rat kidney, liver or regenerating liver, hamster embryo cells [12], human and hamster endometrium [20], thyroid tissue [22], and hamster cardiac muscle, dermis or spleen [13] all failed to induce new capillary growth. On the other hand, some non-neoplastic tissues are capable of stimulating the formation of new vessels. A weakly active angiogenic factor has been isolated from rat and calf thymus and thymic lymphocytes by Folkman and co-workers, and these workers also found a strongly positive vasoproliferative factor in human placenta [12]. Nishioka and Ryan [13] have shown that neonatal hamster epidermis, buccal membrane and, to a lesser extent, lung induced new vessel growth under experimental conditions in which no vascular growth was observed in response to heart muscle, dermis, spleen, and liver (vide supra). Further, this study has shown that implants of adult hamster epidermis, in both normal and inverted orientation, induce a strong vasoproliferative response; whereas dermis, prepared from the same piece of skin, causes no angiogenesis.

That this neovascularization in response to epidermis was not due simply to the mere physical presence of the implant was ruled out by the observation that epidermis inactivated by heating did not cause vascular overgrowth. This experiment further suggested the heat-labile nature of the active component.

Sewell [23] has shown that the presence of certain "inert" materials such as catgut, silk, and synthetic fibers in the chamber may cause tortuosity in the existing vasculature but no new vessel growth. Millipore filter, dialysis membrane, and polythene film, as used in the present study for support or as barriers, when inserted alone caused no vasoproliferation.

A further possible cause of angiogenesis is the

nonspecific vasoinductive effect of inflammation arising either as a result of the surgical procedures carried out on the host hamster or perhaps the liberation from the implant of inflammatory factors produced either by the preparative manipulations or due to autolytic processes occurring within the implant fragment. The former possibility was minimized by the use of strict aseptic techniques in both preparation of the implant materials and during chamber insertion coupled with prophylactic tetracycline. Where possible, test solutions were passed through a bacteriologic filter prior to application to the cheek pouch although of course this procedure would not necessarily remove toxins released by organisms prior to filtration. Heating the implant at 80° C for 30 min inhibited release of the vasoformative factor, yet at the same time promoted autolysis which within 8–10 days had lysed the area of the pouch wall underlying the implant. This feature argues against the neovascular proliferation seen with normal epidermal implants being due to products of autolysis.

Further circumstantial evidence against the epidermal angiogenic factor acting simply through the inflammatory mechanism is offered by the work of Cavallo and colleagues [24] who applied 0.2% formic acid as an inflammatory stimulus and observed a much slower developing and reduced angiogenic effect compared with their TAF. As dermoepidermal separation was accomplished by a concentrated solution (2 M KBr) in this study, the possibility of a chemically induced neovascularization was also considered. That this was not the case was indicated by the fact that, whereas the epidermis subsequently released a vasoformative factor, dermis prepared from the same piece of skin did not. Also, as a further check, pieces of Millipore filter membrane, subjected to 2 M KBr incubation followed by washing, caused no new vessel growth when applied to the pouch membrane.

Folkman and his colleagues [12], after isolating the tumor angiogenic factor from Walker 256 ascites tumor cells, found that it was nondialyzable and was destroyed by heating at 56° C for 1 hr but was stable at 37° C, at room temperature for at least 72 hr, and at 4° C for at least 3 weeks. The activity was destroyed by incubation with ribonuclease or subtilisin but not by trypsin. Several striking similarities between this TAF and the angiogenic factor isolated from epidermis in the present study have emerged. Epidermal factor will diffuse from epidermis and through a microporous filter but will not pass a dialysis membrane. It can be extracted with saline from homogenized epidermis and this extract is still potent following ultracentrifugation and passage through Millipore filter membrane. The factor is inactivated by heating at 56° C for 1 hr or at 80° C for 30 min. It is stable for at least 7 days at 4° C but is lost on protein precipitation of the extract with trichloroacetic acid. After ethyl acetate extraction the angiogenic factor remained in the aqueous phase, arguing against a lipid configuration.

The mechanism of action of these angiogenic factors is of great interest. It is possible that the angiogenic factor derived from epidermis, like Folkman's TAF, can act directly as a mitogen on the vascular endothelium. Alternatively, Ryan and colleagues [2, 25] have demonstrated both proactivators and inhibitors of fibrinolysis in human epidermis and have theorized [13] that deposition of a fibrin scaffold may be important in the mechanics of cutaneous neovascularization. In the present study the implants were found to be encased in a gelatinous mass which histologically appeared as a pale-staining amorphous matrix supporting the newly formed vessels. Histochemical and immunofluorescent staining demonstrated the presence of both fibrin and complement in this amorphous "scaffold."

Thus the epidermis may be acting indirectly by inducing fibrin deposition which in turn provides the requisite environment and stimulus for new vessel growth. An analogous mechanism has been postulated for the invasiveness of malignant tumors. O'Meara [26], demonstrating a labile coagulative factor released from the growing edge of invasive tumors (and which in many respects seems similar to Folkman's TAF), suggests that a fibrin network beyond the tumor periphery is a necessary prerequisite for the establishment of an adequate blood supply to the tumor. Besides its elaboration by neoplastic tissue, this coagulative factor has been found in placenta as well as in chorion, amnion, and colon.

If fibrin deposition is an intermediate step in new vessel production then the addition of fibrin clot to the chamber should mimic the vasoinductive effect of epidermis. Fibrin-impregnated Millipore discs did produce a marked vasoproliferative effect but as the response may have been due, for example, to excess thrombin or to fibrin degradation products, further studies are necessary to implicate fibrin convincingly in the mechanism of action of angiogenic factors. Evidence against the participation of fibrin is provided by the observations [12] that, whereas fibrin depots were induced by TAF which had not been subjected to lipid extraction, no such effect was noted with lipid-free TAF.

These studies have demonstrated the ability of separated hamster epidermis and epidermal extracts to induce neovascularization of the vascular bed of the hamster cheek pouch. The mechanism of action may be similar to that of certain neoplastic tissues in inducing a new tumor blood supply, and the biochemical similarities between the epidermally derived factor and the tumor angiogenic factor as described by Folkman and colleagues are inescapable.

Epidermis, removed from its normal environment may, on application to the cheek-pouch

membrane, find itself under conditions comparable to those seen in wound healing or metastasis formation insofar as the induction of a new blood supply is concerned. The degree of dedifferentiation which occurs may be a sufficient stimulus for the release of a vasoformative factor. One can speculate that certain of the clinical symptoms of psoriasis may be indicative of a failure within the cells of the epidermis in vivo to suppress the formation or release of the angiogenic factor. Folkman [27] has recently theorized along these lines, suggesting that a psoriatic epidermis and capillary endothelial relationship may be regulated by a substance similar to TAF. Others have argued that the rapid cell turnover in psoriasis may be due to a defect in chalone regulation of the epidermal mitotic rate [28–30]. It is tempting to combine these two concepts as a theoretical illustration of the broad implications of an endothelial chalone in the regulation of the cutaneous microvasculature.

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