protein. Ann NY Acad Sci 321:366–381, 1978
29. Kleinman HK, Klebe AJ, Martin GR: Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88:473–485, 1981
30. Carlsen RA: Human fetal hair follicles: the mesenchymal component. J Invest Dermatol 63:206–211, 1974
31. Tarin D, Croft CB: Ultrastructural studies of wound healing in mouse skin II. Dermo-epidermal interrelationships. J Anat 106:79–91, 1970
32. Couchman JR, Rees DA, Green MR, Smith CG: Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry into the division cycle. J Cell Biol 93:402–410, 1982
33. Critchley DR, England MA, Wakely J, Hynes RO: Distribution of fibronectin in the ectoderm of gastrulating chick embryos. Nature 280:498–500, 1979
34. Grinnell F, Billingham RE, Burgess L: Distribution of fibronectin during wound healing *in vivo*. J Invest Dermatol 76:181–189, 1981
35. Clark RAF, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. J Invest Dermatol 79:264–269, 1982

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Biochemical Modulation of Angiogenesis in the Chorioallantoic Membrane of the Chick Embryo

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A variety of substances potentially having effects on angiogenesis in the skin were assayed on the chorioallantoic membrane of the chicken embryo (CAM). Millipore filter discs alone and saturated with saline 0.9% (controls), keratinocyte-conditioned medium, lactic acid 10^{-1} M, adenosine 10^{-4} M, sodium fluoride 10^{-4} M, dinitrophenol 10^{-4} M, histamine 10^{-4} M, 5-hydroxytryptamine 10^{-4} M, acetylcholine 10^{-4} M, prostaglandin E_2 3×10^{-4} M, prostaglandin $F_{2\alpha}$ 3×10^{-4} M, arachidonic acid 10^{-4} M, epidermal growth factor 5×10^{-5} g/ml, human plasma fibronectin 10^{-4} g/ml, acetylsalicylic acid 10^{-3} M, and arachis oil were applied to the CAM and the vascular responses quantitated 4 days later. None of the agents with the exception of keratinocyte-conditioned medium stimulated new vessel growth as compared to the controls. However, arachis oil ($p < 0.001$) and ADP ($p < 0.01$) were associated with significantly decreased vascular responses relative to controls. The specimens incubated with saline, fibronectin, ADP, and arachis oil were examined histologically; with the exception of arachis oil all displayed ectodermal epithelial and mesenchymal hyperplasia of the membrane in association with increased vascularity. Almost no perceptible change was noted histologically with arachis oil.

Despite a great deal of work in the past decade to isolate and characterize substances modulating new blood vessel growth, much remains to be learned concerning the basic mechanisms of angiogenesis. Over this period of time, the list of agents postulated to effect vasoproliferation has continued to grow [1]. Since any factor affecting vascular tone [2], permeability [2], endothelial mitosis (in vitro), or producing tissue or vessel injury [1] may theoretically influence vessel growth, the length of such a list is not surprising. In addition, due to the difficulty in measuring angiogenesis, virtually all studies have used essentially nonquantitative methods of assessing vessel growth, precluding statistical analysis [3].

New vessel growth in the skin may be associated with increased epidermal metabolic demands or inflammatory changes following a variety of insults [2]. Therefore, a host of substances could potentially influence vasoproliferation here as in other circumstances: tissue metabolites or inflammatory mediators released from injured or ischemic epidermis [2], or infiltrating inflammatory cells—mast cells [4], macrophages [5,6], neutrophils [7–9], lymphocytes [10–13], or platelets [14–16]. Some of these agents—lactic acid [17], ADP [18], histamine [19], 5-hydroxytryptamine [19], acetylcholine [19], prostaglandins [20]—have previously been reported to affect new blood vessel growth. In the present work, using a bioassay we have previously developed [21] employing the chorioallantoic membrane of the chicken embryo (CAM), we have reexamined the effects on vasculogenesis of these and other substances relevant to new blood vessel growth in the skin.

MATERIALS AND METHODS

Preparation of Eggs

Fertilized eggs were obtained from local hatcheries, incubated, and processed according to the following protocol slightly modified from Zwilling [22]:

Day 0	Incubation of fertilized eggs at 37°C, approximately 70% humidity; eggs turned automatically every 30 min
Day 3	Two milliliters of albumin aspirated from the narrow end of egg, producing an air sac between the embryo and the shell
Day 4	Small window (approximately 5 mm) created in shell over the air space to prevent CAM from adhering to the shell in this area; window sealed with cellotape and returned to incubator
Day 10	Large window (1–2 cm diameter) made in same area (over

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Abbreviations:
CAM: chorioallantoic membrane (of the chicken embryo)

dropped CAM); materials applied to CAM at this time and window resealed with cellotape
Day 11–13 Eggs examined daily if desired
Day 14 Vascular response quantitated, egg terminated, photographed, and fixed for histologic examination.

Solutions of the substances listed below (Sigma Chemical Company, St. Louis, Missouri) were prepared in physiologic saline (0.9%), neutralized if necessary (pH 7.2–7.4), and filtered (Millipore, 0.22 μm pore size), unless otherwise stated. The concentrations used in these studies were comparable to or higher than those employed in similar investigations [19,20,23]. In almost all instances other concentrations (10^{-3} M, 10^{-5} M, 10^{-6} M) were examined and were without significant difference; epidermal growth factor was examined at one concentration only because of a limited quantity of the agent.

Metabolic products
Lactic acid 0.1 M
Adenosine 10^{-4} M
Adenosine-5-monophosphoric acid (AMP) 10^{-4} M
Adenosine-5-diphosphate (ADP) 10^{-4} M
Adenosine-5-triphosphate (ATP) 10^{-4} M

Metabolic inhibitors
Inhibition of glycolysis - sodium fluoride 10^{-4} M
Inhibition (uncoupling) of oxidative phosphorylation - dinitrophenol 10^{-4} M

Mediators and other agents
Histamine 10^{-4} M
5-Hydroxytryptamine (5-HT) 10^{-4} M
Acetylcholine 10^{-4} M
Prostaglandin E_2 3×10^{-4} M
Prostaglandin $F_{2\alpha}$ 3×10^{-4} M
Arachidonic acid 10^{-4} M
Epidermal growth factor 5×10^{-5} g/ml
Human plasma fibronectin 10^{-4} g/ml (Collaborative Research Inc.)

Other inhibitors
Inhibition of prostaglandin synthesis
Acetylsalicylic acid 10^{-3} M (also applied on day 12)
Reduction in surface activity
Arachis oil (peanut oil; glycerides of: oleic acid 56.0%, linoleic acid 26%, palmitic acid 8.3%, stearic acid 3.1%, etc.) - sterile, applied as is

Two-millimeter Millipore filter discs saturated with substances listed above, were applied to the CAM on day 10. Similar discs applied alone or saturated with saline served as controls. Human epidermal keratinocyte-conditioned medium which has been shown to stimulate angiogenesis in the CAM [27] was used as a "positive" control, i.e., to demonstrate that the CAM was capable of generating an angiogenic response significantly greater than that secondary to carrier materials. Aseptic technique was used at all times. The vascular responses were recorded 4 days later.

Quantitation of the Vascular Response—the "Vascular Index"

The vascular responses (termed the vascular index) were quantitated in ovo using a Wild M5 stereomicroscope (24× magnification) with a fiberoptic light source. All discernible vessels—capillaries, arterioles, venules—traversing a 1-mm annulus about the 2-mm Millipore filter disc were counted provided that they formed an angle of less than 45° with a line radiating from the center (Fig 1). Vessels branching within the annulus were counted as 1 vessel, whereas those branching outside the annulus were considered 2 vessels. Representative responses were photographed on day 14 after fixation in formalin for several hours.

Statistical analysis of the vascular responses was accomplished with Student's *t*-test.

Routine Histologic Preparation

A few representative specimens from control and experimental groups in which fibronectin, ADP, and arachis oil were applied were obtained on day 14 by immersing the egg in 10% phosphate-buffered formalin (pH 7.0). After fixation for at least 24 h, normal and experimental foci were excised, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin (H&E).

RESULTS

Vascular Responses

As indicated in Table I, both the control discs, whether applied alone or saturated with saline, stimulated virtually the same vascular responses. The keratinocyte-conditioned medium generated the only result significantly greater than the controls ($p < 0.001$). Otherwise almost all the substances tested resulted in a radial ingrowth of vessels (Fig 2) not substantially different from that of the controls. In addition, vascular tortuosity was usually present beneath the Millipore discs and correlated in degree with the value of the vascular index (Fig 2). However, two substances, ADP and arachis oil, were associated with statistically significant reduction in the vascular response, with arachis oil producing the greater suppression of growth.

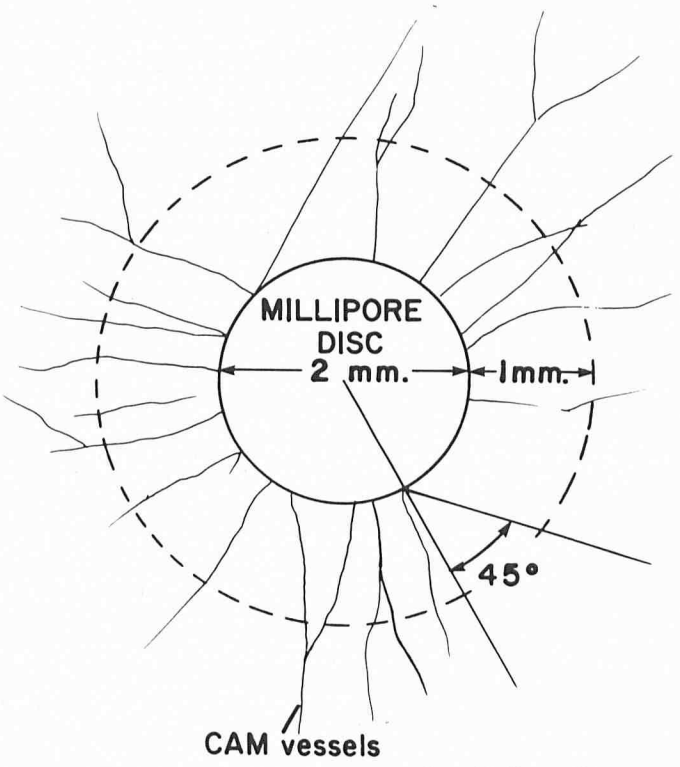

FIG 1. Determination of the vascular index. All discernible vessels traversing the 1-mm anulus about the disc are counted provided they form an angle of less than 45° with a line radiating from the center of the disc (vascular index = 17).

TABLE I. *Vascular responses of the CAM quantitated 4 days after application of various substances*

Substance	Vascular index (Mean ± SD)
Millipore discs (18)[a]	34.8 ± 10.0
Saline 0.9% (65)	33.7 ± 11.7
Keratinocyte-conditioned medium (15)	47.1 ± 11.2[b]
Lactic acid 0.1 M (23)	32.6 ± 12.0
Adenosine 10^{-4} M (11)	29.1 ± 9.3
AMP 10^{-4} M (8)	38.4 ± 7.9
ADP 10^{-4} M (9)	25.4 ± 6.0[c]
ATP 10^{-4} M (10)	33.0 ± 15.8
Sodium fluoride 10^{-4} M (9)	34.1 ± 17.5
Dinitrophenol 10^{-4} M (10)	33.8 ± 4.2
Histamine 10^{-4} M (12)	34.5 ± 9.9
5-HT 10^{-4} M (11)	34.6 ± 13.0
Acetylcholine 10^{-4} M (10)	32.3 ± 7.4
Prostaglandin E_2 3×10^{-4} M (9)	33.1 ± 9.7
Prostaglandin $F_{2\alpha}$ 3×10^{-4} M (12)	31.7 ± 14.6
Arachidonic acid 10^{-4} M (13)	37.7 ± 11.1
Epidermal growth factor 5×10^{-5} g/ml (12)	31.6 ± 10.7
Fibronectin 10^{-4} g/ml (19)	38.3 ± 10.2
Acetylsalicylic acid 10^{-3} M (11)	35.0 ± 10.8
Arachis oil (11)	18.7 ± 10[b]

[a] Numbers in each group given in parentheses.
[b] $p < 0.001$ vs controls.
[c] $0.001 < p < 0.01$ vs controls.

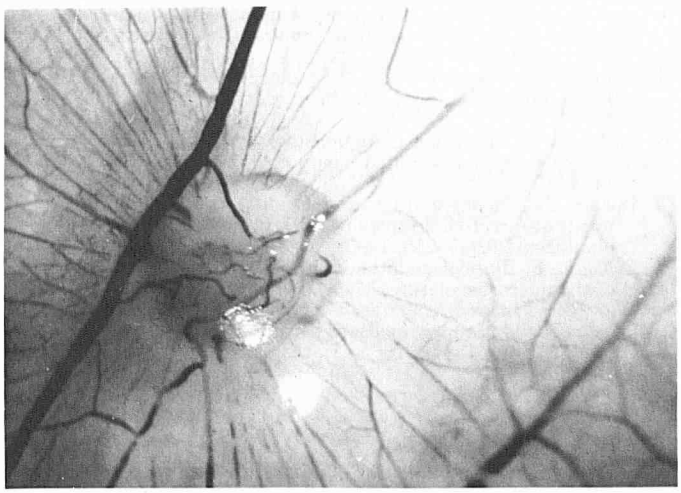

FIG 2. The vascular response of the CAM after 4 days (day 14) to a 2-mm Millipore filter disc saturated with physiologic saline. There is radial ingrowth of vessels with a vascular index of 35. The undersurface of the disc shows increased tortuosity of vessels (× 4).

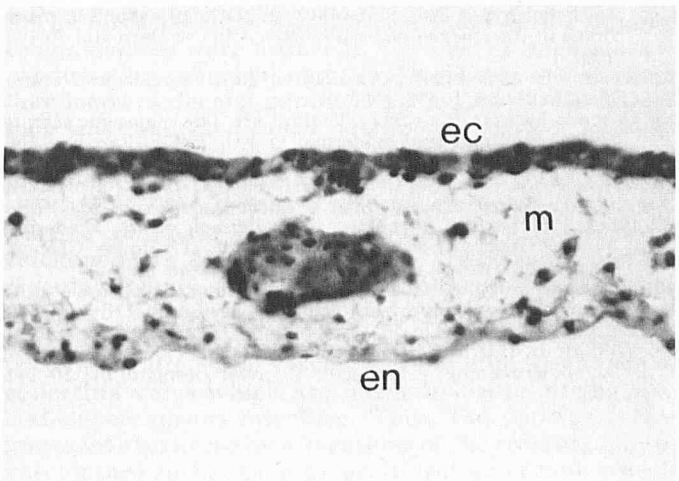

FIG 3. Cross section of the normal unstimulated chorioallantoic membrane (day 10). The middle mesenchymal vascular layer (*m*) is sandwiched between the upper ectodermal epithelium (*ec*) and the lower endodermal epithelial layer (*en*). A medium-sized vessel can be seen in the center of the mesenchymal layer (H&E, × 400).

Histologic Responses

The normal unstimulated CAM is shown in Fig 3. The application of a saline-saturated Millipore filter disc for 4 days resulted in significant hyperplasia of the ectodermal and mesenchymal layers with an obvious increase in the number of vessels (Fig 4). Leukocytic infiltration was negligible or minimal (Fig 4). Similar changes were noted in the specimens examined from groups treated with ADP and fibronectin. However, the group in which arachis oil was applied demonstrated almost no change in the membrane when compared with the normal unstimulated CAM. Epithelial and mesenchymal proliferation was negligible.

DISCUSSION

A number of chemical substances potentially having effects on vascular growth in the skin were studied in the present work. In contradistinction to previous studies [17–20,23], there was no evidence that lactic acid, ADP, histamine, 5-hydroxytryptamine, acetylcholine, prostaglandins, or epidermal growth factor promoted endothelial growth beyond that occurring secondary to saline-saturated discs. A major reason for the disparity between our findings and previous studies almost certainly was related to differences in the method of measuring vessel growth. As already mentioned, because of the difficulty in quantitating angiogenesis, previous investigators have generally employed techniques not amenable to statistical analysis, and consequently it has been impossible to know whether significant differences existed. Obviously our method has differed from previous ones by being completely quantitative.

A potential criticism of the data was that the baseline level of angiogenesis produced by the Millipore filter discs might obscure or mask the vessel growth stimulated by any one of these agents. We cannot deny this possibility but can only point out that the degree of vascular response to the discs was relatively low and consequently would mask only a low level of angiogenesis [26]. Virtually anything applied to the CAM might engender a response of this magnitude nonspecifically [3]. To further emphasize this point, we have demonstrated a significantly greater vascular response secondary to keratinocyte-conditioned media compared to controls and the experimental groups; this indicated that the CAM was indeed capable of generating substantial vasoproliferation, clearly greater than that resulting from carrier materials. Similar data with other substances have been noted by others [26,28].

Because of the relatively rapid dissipation of the substances from the Millipore discs, probably over several hours, another possible criticism of the assay was that a longer period of exposure to (or release of) an angiogenic factor might be necessary for initiation of neovascularization. This was undoubtedly a worthy point to consider, but it must be stressed that apparently valid results have been generated in other studies using similar methodology—a single application of the substances assayed [26].

The significance of the diminution of vessel growth secondary to ADP was unclear. An obvious interpretation was that this substance had an adverse rather than a potentiating effect on cell growth. Various products of cellular metabolism have in fact been postulated to inhibit proliferation [24], and it is therefore possible that epithelial and/or endothelial growth may have been partially suppressed through this mechanism. However, the histologic changes did not point to a major effect on epithelial hyperplasia.

Arachis oil also suppressed angiogenesis and this was most likely secondary to a reduction in surface-free energy or activity of the Millipore filter discs. The effects of hydrophobic (or-

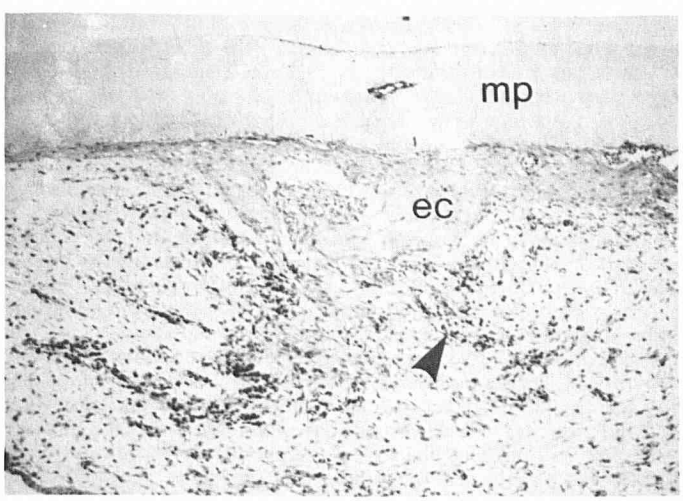

FIG 4. The CAM 4 days (day 14) after application of a saline-saturated Millipore filter disc (*mp*). There is hyperplasia at the ectodermal epithelium (*ec*) and mesenchyme as well as increased numbers of vessels (*arrowhead*) (H&E, × 100).

ganic) substances in the diminution of surface activity have been well documented [25]. This would almost certainly result in decreased cellular adhesion and in turn, decreased epithelial proliferation and possibly reduction in inflammatory changes as well. Since ectodermal epithelial hyperplasia is probably the major stimulus to angiogenesis secondary to disc application (inflammation being relatively minimal) [27], suppression of epithelial hyperplasia would have removed the predominant stimulus to new vessel growth. Histologic findings supported this notion since the various components of the membrane were of essentially normal thickness.

In conclusion, we have demonstrated that a variety of substances relevant to vessel growth in the skin did not promote angiogenesis as examined here. It is likely that many previous positive results with a number of these agents may have arisen from the failure to use quantitative methods in measuring new vessel growth.

REFERENCES

1. Auerbach R: Angiogenesis—inducing factors: a review, Lymphokines, vol 4. Edited by E Pick. New York, Academic Press, 1981, pp 69–88
2. Ryan TJ: Factors influencing the growth of vascular endothelium in the skin. Br J Dermatol 82(suppl 5):99–111, 1970
3. Jakob W, Jentzsch KD, Mauersberger B, Heder G: The chick embryo chorioallantoic membrane as a bioassay for angiogenesis factors: reactions induced by carrier materials. Exp Pathol (Jena) 15:241–249, 1978
4. Kessler DA, Langer RS, Pless NA, Folkman J: Mast cells and tumor angiogenesis. Int J Cancer 18:703–709, 1976
5. Polverini PJ, Cotran RS, Gimbrone MA, Unànue ER: Activated macrophages induce vascular proliferation. Nature 269:804–805, 1977
6. Nathan CF, Murray HW, Cohn ZA: The macrophage as an effector cell. N Engl J Med 303:622–626, 1980
7. Fromer CH, Klintworth GK: An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. Am J Pathol 79:537–554, 1975
8. Fromer CH, Klintworth GK: An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. II. Studies on the effect of leukocytic elimination on corneal vascularization. Am J Pathol 81:531–544, 1975
9. Fromer CH, Klintworth GK: An evaluation of the role of leukocytes in the pathogenesis of experimentally vasoproliferative capability of polymorphonuclear leukocytes and lymphocytes. Am J Pathol 82:157–169, 1976
10. Sidky YA, Auerbach R: Lymphocyte-induced angiogenesis: a quantitative and sensitive assay of the graft-vs.-host reaction. J Exp Med 141:1084–1100, 1975
11. Auerbach R, Kubai L, Sidky Y: Angiogenesis induction by tumors, embryonic tissues, and lymphocytes. Cancer Res 36:3435–3440, 1976
12. Nishioka D, Katayama I: Angiogenic activity in culture supernatant of antigen-stimulated lymph node cells. J Pathol 126:63–69, 1978
13. Pliskin ME, Ginsberg SM, Carp N: Induction of neovascularization by mitogen-activated spleen cells and their supernatant. Transplantation 29:255–258, 1980
14. D'Amore P: Platelet-endothelial interaction and the maintenance of the microvasculature. Microvasc Res 15:137–145, 1978
15. Wall RT, Harker LA, Striker GE: Human endothelial cell migration. Stimulation by a released platelet factor. Lab Invest 39:523–529, 1978
16. Zetter BR, Antoniades HN: Stimulation of human vascular endothelial cell growth by a platelet-derived growth factor and thrombin. J Supramol Struct 11:361–370, 1979
17. Imre G: The mechanism of corneal vascularization. Acta Morphol Acad Sci Hung 14:99–104, 1966
18. Fraser RA, Ellis EM, Stalker AL: Experimental angiogenesis in the chorioallantoic membrane (abstr). Bibl Anat 18:25, 1979
19. Zauberman H, Michaelson IC, Bergmann F, Maurice DM: Stimulation of neovascularization of the cornea by biogenic amines. Exp Eye Res 8:77–83, 1969
20. BenEzra D: Neovasculogenic ability of prostaglandins, growth factors, and synthetic chemoattractants. Am J Ophthalmol 86:455–461, 1978
21. Barnhill RL, Ryan TJ: Quantification of neovascular responses to epithelial hyperplasia and injury in the chorioallantoic membrane of the chicken embryo (abstr). J Invest Dermatol 76:428, 1981
22. Zwilling E: A modified chorioallantoic grafting procedure. Transplant Bull 23:115–116, 1959
23. Gospodarowicz D, Bialecki H, Thakral TK: The angiogenic activity of the fibroblast and epidermal growth factor. Exp Eye Res 28:501–514, 1979
24. Shymko RM, Glass L: Cellular and geometric control of tissue growth and mitotic instability. J Theor Biol 63:355–374, 1976
25. Baier RE: Surface chemistry in epidermal repair, Epidermal Wound Healing. Edited by HI Maibach, DT Rovee. Chicago, Year Book Medical Publishers, 1972, pp 27–45
26. Klagsbrun M, Knighton D, Folkman J: Tumor angiogenesis activity in cells grown in tissue culture. Cancer Res 36:110–114, 1976
27. Barnhill RL: M.Sc. Thesis, University of Oxford, 1982
28. Wolf JE Jr, Harrison RG: Demonstration and characterization of an epidermal angiogenic factor. J Invest Dermatol 61:130–141, 1982