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Reviews

Tumor-derived angiogenesis factors from rat Walker 256 carcinoma: an experimental investigation and review[1]

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Summary. Angiogenesis, the process of developing a hemovascular network, is an essential feature of the growth of solid tumors, and is induced by factors secreted by tumor cells. Assay procedures suitable for the investigation of angiogenesis, and for the screening of angiogenesis factors during purification are reviewed; and a number of reports describing the purification of angiogenesis factors, primarily from the rat Walker 256 carcinoma as starting material, are discussed. Work from the authors' laboratory is also presented. Walker 256 cells grown in large-scale culture were the source of a reproducible and homogeneous source of angiogenic material. Factors secreted by these cells were isolated by a series of chromatographic steps. Ion exchange chromatography on carboxymethyl-Sephadex produced two active fractions, one of which was fractionated into several macromolecular species by lectin affinity and hydrophobic adsorption chromatography. The other gave a high mol.wt, active fraction that was resolved into a low mol.wt, active component and a non-angiogenic but possibly carrier molecule with a mol.wt of 140,000. While none of the angiogenic factors were identified chemically, the results demonstrate the existence of both high and low mol.wt tumor-secreted angiogenic substances, confirming the hypothesis for tumor-induced angiogenesis and predicting potential means to interfere with the process of tumor growth.
Key words. Rat carcinoma; carcinoma, rat; angiogenesis factors, tumor-derived; Walker 256 carcinoma.

1. Introduction

Organogenesis, the process by which cells aggregate, differentiate, and form discrete tissues and organs, was originally addressed through the pioneering efforts of Baltzer, Needham, Spemann and others[10, 64, 78, 88]. Baltzer was probably the first to clearly demonstrate the role played by specific, chemically discrete substances in mediating organ development[10]. He anticipated that many of the questions posed by his contemporary comparative embryologists might be answered by defining the chemical identity of these substances. Some 50 years have elapsed since the publication of these classic works but the questions they raised regarding the chemical nature of mediators involved in organogenesis, their possible mechanisms of interaction with target cells, and the regulatory processes that control their activities remain largely unanswered.

Angiogenesis, the development of a hemovascular network, is a typical example of mammalian cellular differentiation, and owing to the distinct structure of the resultant organ, provides an appropriate system to investigate the identity and mode of action of such soluble mediators or 'molecular messengers'.

Neovascularization occurs during embryonic growth and wound healing, in physiological events such as the cyclical development of the uterine endometrium, and in a number of pathologic states, particularly tumor growth[25]. Indeed the proliferation of blood vessels in the vicinity of solid tumors was described by Virchow more than a century ago[86], and over the years this phenomenon has attracted much attention and experimental effort. By 1907, Goldman had noted that tumor cells induce vascular proliferation and that these new blood vessels, in turn, promote tumor growth[34]. Algire and Chalkley[5] first emphasized that growing tumor cells are associated with a blood vessel growth stimulating factor, and that they continually elicit the formation of new capillaries from the host. These important observations were followed by those of Greenblatt and Shubik[38] and Ehrmann and Knoth[18] who concluded that tumor-induced neovascularization is mediated by a diffusible substance(s). Furthermore, it was found that experimental tumors deprived of a blood supply cannot grow beyond a certain miniscule size, do not metastasize, and may even regress completely[25] ascribing more than one critical function to their vasculature. If the existence of angiogenic mediators could be verified, their isolation, identification, and chemical and functional characterization would provide not only decisive inroads to the study of organogenesis but also have profound clinical implications.

This review examines the assays available for tumor-induced angiogenesis, describes the reports of several laboratories on the isolation and partial characterization of tumor-derived angiogenic factors (TAFs), and presents a summary of our own work on rat Walker 256 carcinoma which was carried out before we turned our efforts to the isolation of TAFs from human sources.

2. *Assays for tumor-induced angiogenesis*

Assays for tumor angiogenesis are of two general types. The first utilizes implants of tumor fragments or cell suspensions to provide reproducible capillary ingrowth from an existing vascular bed, on the one hand allowing detailed morphological and histological examination of tumor-induced neovascularization, and on the other providing a system within which antagonists such as antibodies and 'anti-angiogenesis factor' substances may be evaluated. The second uses implants of soluble, tumor cell-derived extracts in a variety of forms to screen for the presence of TAF, e.g. during the course of isolation.

The earliest work that specifically examined tumor-induced angiogenesis used the transparent chamber technique. Originally designed by Sandison[72] and Clark et al.[16] to investigate new blood vessel growth during wound healing in the rabbit ear, it was modified by Algire and Chalkey[4,5] for insertion in the mouse back. This allowed the investigation of angiogenesis caused by the many transplantable mouse tumors. These classic studies[4,15] not only indicated the marked degree of neovascularization induced by a variety of tumor implants, but drew attention to the possibly critical role of angiogenesis in tumor growth and development. In 1964, a further chamber modification was developed[71] for use in the Syrian hamster cheek pouch, which provided two major improvements: back illumination from within the mouth and – since the cheek pouch is an immunologically privileged site[77] – examination of tumors from other species. The use of these chamber techniques led to three general conclusions. First, virtually all tumor tissues studied induce neovascularization. Second, angiogenesis occurs by budding from existing host capillaries in a fashion analogous to that previously described for wound healing; and third, both the time of emergence and the final structure of tumor-induced blood vessels vary greatly[77].

One disadvantage of these assays is that tumor implants are placed within an existing vascular bed. In order to develop a system in which the tumor was separated from the vascular bed, a modified version of the rat dorsal air sac technique of Selye[76] was employed by Folkman and coworkers[25]. In this technique air is injected s.c. and the skin lifted away from an area of poorly vascularized fascia. When rat Walker tumor cells enclosed in Millipore chambers were implanted into these air pockets, a vascular response could be observed beneath the filter. Histologic and autoradiographic studies revealed endothelial mitoses.

Detailed ultrastructural autoradiographic studies carried out with this same animal model[14] showed that endothelium had begun to regenerate within 48 hours and was accompanied by a marked increase in ribosomes and endoplasmic reticulum, scarce or absent pinocytotic vesicles, and a discontinuous basement membrane. Mitotic endothelial cells were present along the newly formed capillary sprouts as well as in the parent vessels. These studies were important in that they provided clearer evidence for endothelial cell proliferation and new capillary sprouts in response to tumor cells or tumor extracts, and because they showed proliferative responses several mm from the nearest tumor cell. However, this technique also has several limitations; in particular vascular growth can neither be quantitated nor observed continuously by this means.

The development of methods for the implantation of tumor and other tissue in the avascular corneal stroma of the rabbit eye constituted a major breakthrough in the field[30]. Although the eye had been used as a site for tumor transplantation[39,40], it had not served for tumor angiogenesis assays. By locating the tumor in the cornea at a suitable distance from the vascular bed, new vessels grow out from and perpendicular to the existing vessels of the limbus, allowing a quantitative evaluation of neovascularization. New Zealand White rabbits, 4–6 months old, are given i.v. anesthesia supplemented by corneal application of a few drops of xylocaine. The eyeball is proptosed and a 1.5-mm incision is made on the corneal dome to about one-half depth of the cornea. A pocket is produced in the cornea with an iris spatula, allowing the insertion of a fragment of tumor tissue. The bottom of the pocket should be located no more than 2 mm from the limbus since the angiogenic stimulus is not effective over larger distances.

In the initial studies fragments of Brown-Pearce and V2 rabbit carcinomas were implanted into the avascular corneal stroma of rabbits at distances from the limbus of 1–6 mm[14]. Tumor growth and neovascular response of limbal vessels were studied by slit-lamp stereomicroscopy, histologic examination, colloidal carbon injections, and autoradiography. Tumors placed 5–6 mm from the limbus spread as thin plates toward the limbus. When the tumor edge reached to within about 2 mm of the limbus, new vessels began to grow from the limbal plexus toward the tumor. Once penetrated by new capillaries, the tumor grew rapidly. Immunologically incompatible mouse tumors elicited new vessels in a similar way. However, after vascularization, immunological rejection occurred.

While the rabbit cornea is used most widely, these methods have been modified for use in guinea pigs[68], rats[29], and mice[62,63], and a wealth of detailed histological and morphological information on tumor-induced angiogenesis in the eye is now available[8,9,63].

For the screening of angiogenic activity in tumor extracts only a limited number of suitable bioassays have been developed. In the early studies of TAF by Folkman and coworkers[27], test fractions were injected intermittently over a two-day period into the s.c. dorsal air sac of the rat through an implanted silicone rubber tube. The fascia was then examined for neovascularization after anesthesia. This method, no longer used because it is both cumbersome and requires large quantities of material, pointed to the different problems

involved in screening samples of TAF activity as opposed to examination of the effects of tumor fragments. In particular, the need for many assays, and for the continuous presence of material over a long enough period of time to sustain blood vessel ingrowth, have required new approaches.

Large numbers of samples can be screened by the method of Knighton et al.[50] which uses the chick chorioallantoic membrane (CAM). The assay has been described in detail elsewhere[42,50]. Briefly, it involves aspirating 1 ml of albumin from 3-day-old fertilized chicken eggs thereby allowing the CAM to drop away from the shell. The CAM is then exposed by cutting a 5-cm^2 window through the shell on day 4 or 5. A small amount of lyophilized sample is applied to the CAM from the tip of a spatula at day 9. Activity is monitored with a microscope for 7 days and expressed as negative or positive [1+ (weakest) to 4+ (strongest)] depending on the degree of angiogenic response.

Recently, a more quantitative and reproducible modification of this procedure developed by Folkman has been adopted by many laboratories[42]. Samples to be tested (micro- to nanogram amounts) are dissolved in sterile, distilled water and 5-µl aliquots are placed on transparent 13-mm plastic discs (Thermanox, Flow Labs), dried, and the inverted discs placed on the 9 day CAM. Responses are assessed after 1, 2, and 3 days and recorded as the number of positive results per number of eggs implanted per sample. Usually 4 or 5 eggs are used for each concentration of sample tested. Samples are designated 'active' if positive responses in the absence of gross inflammation are seen in at least 60% of the eggs on day 3. Positive responses can be examined histologically after formalin fixing of the CAM.

A variety of other means of sample implantation on the CAM have been used, including glass fiber and nitrocellulose filters[49,83], glass rings[79], methylcellulose[79] and lactose[66,87]. In all methods a characteristic, white infiltrate beneath the disc diagnoses the existence of gross inflammation (which is accompanied by angiogenesis). The main drawback of the CAM assay is its sensitivity to a variety of stimuli which results in this false positive inflammatory response[46]. *A single, positive response can never be accepted as evidence for true angiogenesis. This requires multiple assays and a dose-response curve over a range of sample concentrations.*

In general, samples deemed positive by CAM screening should be tested in the corneal implant assay. Although direct injection of solutions into the cornea with a 30-gauge needle has been used[12,42,47], a more stringent method of 'sustained release' is often desirable and is usually achieved by incorporation of the sample into an inert, noninflammatory polymer. This has been studied extensively[54] and the polymers most widely used are Elvax 40 (DuPont), an ethylene-vinyl acetate copolymer (40% vinylacetate by weight) and Hydron (Hydron Labs), a polyhydroxymethacrylate polymer. It is essential to wash the Elvax polymer exhaustively in ethanol at 37 °C to remove inflammatory contaminants. Once clean, a 10% casting solution of Elvax 40 is prepared in methylene chloride and mixed with the dry test substance under aseptic conditions. The mixture is quickly frozen to avoid settling and the solvent is evaporated under vacuum. The dry pellet is cut into 1-mm^3 pieces and implanted into the cornea in a fashion analogous to that employed for tumor fragments. Sustained release occurs over a period of many days[54]. The Hydron polymer requires no pretreatment, and is dissolved as a 10–12% solution in 95% ethanol at 37 °C. This solution can be mixed directly with powdered sample, or with an equal volume of sample in sterile water, and pellets formed and implanted as for Elvax. Experience suggests that Hydron pellets are somewhat less satisfactory than Elvax pellets[42], producing a greater percentage of nonspecific inflammatory side reactions.

One other assay has had limited use. McAuslan and coworkers examined extracts from bovine parotid or mouse submaxillary glands for angiogenic capacity by injecting them into mice s.c.[59,60]. They observed capillary endothelial cell proliferation in a variety of organs but particularly in the kidney. Their initial fractionation of an angiogenesis factor was monitored by this method. However, since this assay requires a relatively large amount of material and is not quantitative, it has been superceded by more conventional approaches[59]. Moreover, the assay is likely artifactual, reflecting side effects of induced glomerular nephritis.

In order to obviate some of the difficulties involved in biological assays for angiogenesis, efforts have been made to correlate angiogenesis in vivo with endothelial cell function in vitro. Thus, after it became clear that tumor products could enhance endothelial cell growth in vitro[6,21,80], a number of laboratories attempted to correlate such stimulation with the angiogenic activity of tumor extracts[22,75]. Unfortunately, there is no universally accepted procedure for assessing endothelial cell growth stimulation in vitro, owing to a number of problems. First, there is a lack of agreement on the correct target cell for in vitro assays, because of the clear differences between the endothelial cells of large vessels and microvessels, both in vivo and in vitro[90]. Second, the substratum upon which endothelial cells rest plays a critical role in their response to growth factors in vitro[35]. Thus, a partially purified TAF from one laboratory induces endothelial cell proliferation in vitro on collagen but not on plastic[75].

Differences have also been found between capillary and aortic endothelial cells in their migratory response to tumor products[48]. Since endothelial cell migration is an early event during tumor-induced angiogenesis[8], it has been suggested that migration rather than proliferation might be a more appropriate in vitro assay for angiogenesis factors[59,89].

A further complication is that endothelial cells form two morphological variants in vitro. Aortic endothelial cells have been shown to form 'sprouts' or lace-like patterns beneath the confluent monolayer, which have been likened to neovascular budding in vivo[17,37,58], while capillary endothelial cells have been shown to form tube-like structures, a phenomenon called 'angiogenesis in vitro'[26]. Originally suggested to be specific for capillary endothelial cells[26,57], in vitro tube formation has now been observed with endothelial cells from both aorta[20,65] and umbilical vein[52,56]. The relevance of either of these in vitro phenomena, 'sprouting' and tube formation, to in vivo neovascularization, the problem of

the role of cell substratum, and of target cell migration versus proliferation, as well as the possibility of yet other in vitro correlates[41], have made in vitro angiogenesis assays a major field of investigation in and of itself.

In summary, none of the procedures available for in vivo angiogenesis are ideal. It is clear that the design and verification of specific, reliable, repeatable and precise methodology to determine angiogenesis in vivo remains an imperative of the highest priority. It is also clear that in vivo TAF activity must be rigorously correlated with in vitro function, either of endothelial cell behavior or any other potential activity, before in vitro angiogenesis assays can be used routinely. Moreover, in view of the broad spectrum of in vitro correlates that have been suggested as well as of the TAF(s) that have been partially purified (see below), it is most unlikely that any in vitro assay will reflect the activity of more than a small fraction of angiogenesis factors relevant in vivo.

3. Isolation of tumor angiogenesis factor: summary of published work

The first attempts to isolate an angiogenesis factor from solid tumor homogenates were made by Folkman and coworkers. They obtained a fraction from Walker 256 carcinoma with a mol.wt of about 100,000 and containing approximately 25% RNA, 10% protein, 50% carbohydrate and a residue probably constituted by lipids. Its angiogenic activity was destroyed by digestion with bovine pancreatic ribonuclease, by heating for 1 h at 56 °C, or by incubation with subtilisin, but the activity was not diminished after 3 months at 4 °C or by exposure to trypsin for up to 3 days[27]. Cell nuclei were also found to contain activity, and in a more detailed study, Tuan et al.[84] demonstrated that the nonhistone protein fraction from the nuclei of Walker 256 carcinoma cells could induce angiogenesis in the rabbit cornea and mitoses in nearby endothelium. Cells were disrupted by N_2 cavitation, nuclei were isolated, and chromatin was purified by differential centrifugation. The chromatin proteins were separated from DNA by chromatography on Bio-Gel A 5m in the presence of 4 M guanidine·HCl and the histones and nonhistones were further resolved on CM-Sephadex C-50. The histone fraction showed no activity in the corneal bioassay, but the nonhistone protein fraction was strongly angiogenic, and the authors concluded that tumor angiogenesis factor was therefore associated with this material.

Subsequent studies revealed that TAF was secreted by a variety of tumor cell lines grown in culture[49]. Folkman summarized the work from his laboratory thus: 'TAF resembles a nondialysable ribonucleoprotein of approximately 100,000 mol.wt. It is found in the tumor cell nucleus associated only with the non-histone proteins. It has also been isolated from tumor cytoplasm and from material which diffuses out of intact tumor cells'[23]. Phillips and coworkers have partially purified TAF from rat Walker 256 carcinoma solid tumors[66, 67, 87]. They isolated a low mol.wt ($\sim$ 200) nonprotein component having strong angiogenic activity on the chick CAM as well as a mitogenic effect on endothelial cells in culture[75]. Walker 256 carcinoma solid tumors were homogenized and extracted following the procedure of Folkman et al.[27] but omitting the trypsin step, and the crude preparation was subjected to gel filtration on Sephadex G-100. Activity was detected in the high mol.wt fraction and subsequently chromatographed on DEAE cellulose using a convex salt gradient between 0 and 0.3 M NaCl. An immunoaffinity column using antibody raised against a crude preparation of angiogenesis factor served to purify the material further. The bound fraction was eluted with 50 mM ammonium acetate, pH 3.7, and applied to a Bio-Gel P-2 column in 10% isopropanol. The angiogenic peak emerged at a volume corresponding to a M_r $\sim$ 200. The chemical nature of the small molecule was not defined, but was reported not to be a prostaglandin, polypeptide, or nucleic acid[87]. Using the same methods a similar or identical material was obtained from synovial fluid[13], other nontumor sources[43, 51] and from the culture medium of a human lung tumor cell line that had been adapted to grow in serum-free medium[52]. The chemical identity of this material remains undefined.

McAuslan and coworkers have partially purified an angiogenesis factor from several sources, including rat Walker carcinoma cells grown in culture[44, 59, 60]. This factor, initially obtained from extracts of bovine parotid and murine submandibular glands, was named 'endothelium stimulating factor' owing to its capacity to induce proliferation of renal capillaries following s.c. injection of both newborn and adult mice[44]. This activity was observed later with crude extracts of Walker 256 carcinoma cells[60]. These workers also isolated fractions from Balb/c 3T3 cells which exhibit the same activity[59]. The cultures contain a polypeptide of M_r 120,000 with mitogenic activity toward bovine aortic endothelium and a low mol.wt (3000 or 210) factor lacking this activity but capable of stimulating angiogenesis in vivo. The 3000 mol.wt fraction, having a pI of 7.8 by isoelectric focusing, consisted of both an inert carrier and the lower mol.wt (210), heat stable angiogenesis factor. Inorganic analysis by spark source mass spectrometry of this second fraction identified zinc and copper as the only two trace elements present in high concentration; 2000 and 8000 ppm, respectively. According to these investigators, only copper salts induced endothelial migration, as did the crude extracts of bovine parotid gland, the fractions from Walker 256 carcinoma, or the material from Balb/c 3T3 cells. Corneal assays confirmed the activity data, and copper complexes were concluded to account for the angiogenic activity from all sources tested, including rat Walker 256 carcinoma. However, as indicated in the previous section, the renal assay likely reflects inflammatory complications of glomerular nephritis. In addition, it is established that copper is chemotactic for leukocytes and thereby involved in many inflammatory pathways, and that copper implanted in the vitreous cavity of the rabbit eye will elicit an acute inflammatory response within 24 h[70]. Moreover, we have examined the effects of copper salts on the chick CAM, and found no angiogenic activity, except at nonphysiological levels, where an intense inflammatory response occurs.

Fenselau and coworkers[22] have also reported the purifi-

cation of an angiogenic substance from the Walker 256 rat tumor, maintained in ascites, which is mitogenic for fetal bovine aortic endothelial cells in culture and stimulates new blood vessel growth in vivo. Lyophilized crude tumor cell homogenate was extracted with absolute ethanol, and the extract was further purified by chromatography on silica gel. The most highly purified material eluted with ethyl acetate:methanol (25:1) and behaved as a single component when analyzed by either thin layer or reversed phase (C_{18}) high performance liquid chromatography. Bio-Gel P-30 chromatography indicates a M_r of 400–800 for the active material whose chemical identity remains to be determined. Its angiogenic activity was established in both the chick CAM and the rat corneal micropocket bioassays. In addition, the purified substance exhibited mitogenic activity toward vascular endothelial cells cultured in both serum-free and serum-containing media.

Certain defined substances which are known to be produced by tumors have been implicated in angiogenesis induction. For example, it has been suggested that prostaglandins of the E series may play a role in the angiogenic response[7, 11, 28, 63, 91]. A highly purified polypeptide, human urokinase, has also been shown to induce neovascularization in the rabbit cornea[12, 33]. However, no careful studies have been carried out that demonstrate any correlation of angiogenic activity with the levels of such factors during purification of TAFs from a tumor source.

In summary, published work points to a variety of soluble mediators that might be involved in the induction of neovascularization. It is difficult to discern the chemical identity of the material obtained in each of these separate studies or, for that matter, any correspondence between them. There are a number of reasons for this. First, sources have varied. Although the rat Walker carcinoma has been used in most cases, it has been used as ascites, solid tumor, or tissue culture. Solid tumors provide rather poor sources because they contain a high proportion of host tissue and cells, particularly macrophages, which may give rise to misleading results. Second, in certain cases careful examination of the available data implicate inflammatory mediators in the observed responses. The suggestion that copper plays a role in neovascularization[59, 69, 91] is a case in point, and the finding of a low mol.wt angiogenesis factor identical to one form of TAF in the synovial fluid of inflamed joints[13] represents another. Third, where defined molecules such as prostaglandins and urokinase have been implicated, no one has carefully followed both the defined molecule and the angiogenic activity during purification of the latter from a crude tumor source and unequivocally demonstrated a correlation. It is clear that a major effort must be made to critically examine, confirm, and correlate current factors thought to be involved in the induction of tumor angiogenesis.

4. Summary of work on Walker 256 carcinoma from this laboratory

4.1 Materials and methods

Mouse submaxillary glands were obtained from adult, male Swiss Webster mice (Pel-Freez Biologicals, Rogers, AR) and extracted according to published procedures[73, 85].

Tumor-derived angiogenesis factors were obtained from rat Walker 256 carcinoma cells that were grown in roller bottles in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). After the cells reached confluency growth medium was removed and cells were washed twice with Ringer's lactate solution at 37 °C and then incubated in this solution for a further 24 h. The resultant extract was dialyzed four times against 10 volumes of water and lyophilized.

For subsequent large-scale work, the Walker 256 cells were adapted for growth in suspension culture and propagated routinely in 12–40 l spinner flasks in DMEM supplemented with 5% FBS. This procedure was performed at the cell culture laboratories of the Monsanto Co., St. Louis, MO[42a]. Cells were harvested at a density of 10^6 per ml, washed with PBS and incubated in $\frac{1}{20}$ volume of this buffer for 4 h at 4 °C. The resultant cell-free, tumor conditioned extract was concentrated and dialyzed four times against 10 volumes of water and lyophilized.

The harvested cells were also examined for the presence of angiogenesis factors. They were lysed by osmotic shock in water at 4 °C, cell debris was removed by centrifugation and the resultant lysate solution dialyzed against water as above and lyophilized.

Samples were prepared for assay of angiogenic activity by extensive dialysis against sterile water in 6000–8000 dalton cutoff membranes (Spectropor) followed by lyophilization. Water was prepared by deionization and then distillation (Barnstead model A10313) into a sterile holding tank irradiated with short wavelength UV light. It was essential to use pyrogen-free, sterile water to avoid false inflammatory CAM responses.

Neovascularization activity was evaluated on the chick CAM as described above. During the early stages of the work the method of Knighton et al.[50] was used (CAM method A), but later the semi-quantitative modification of this procedure[42] (CAM method B) was adopted.

Lyophilized samples were also incorporated into inert controlled-release polymer pellets (Elvax) and assayed after implantation in the rabbit cornea[54]. The length and number of blood vessels growing from the limbus toward the polymer pellet were recorded daily for 14–20 days.

SDS-PAGE was performed as described[53]. Analytical isoelectric focusing was performed on a LKB 2117 multiphor unit using precast PAG plates (LKB) while preparative methods were carried out in Ultrodex (LKB) according to the manufacturer's instructions. Protein concentrations were determined by the method of Lowry et al.[55].

Antisera to partially purified preparations were obtained by inoculating the foot pad of rabbits with 0.5-ml aliquots of lyophilized powder emulsified in complete Freund's adjuvant. The immunoglobulin fractions were isolated from immune serum by DEAE-cellulose chromatography and coupled covalently to cyanogen bromide-activated Sepharose-4B (Pharmacia Fine Chemicals) according to standard protocols.

The stability of TAF in crude Walker 256 extracts was

examined as follows: A) lyophilized extract (300 mg) was suspended in 12 ml of water, mixed, and clarified by centrifugation (30,000 × g, 10 min). Aliquots of 1 ml were mixed with 5 ml of universal buffer[61] and adjusted to a constant ionic strength and final pH of 1.95, 2.90, 3.74, 4.71, 5.69, 6.70, 7.75, 8.64, 9.43, 10.21 and 11.75. After incubation at 37 °C for 1 h, each sample was dialyzed for 20 h at 4 °C against 1 l of NaCl of conductivity equal to that of the buffer and then dialyzed extensively against water. Each sample was centrifuged (20,000 × g, 10 min) and the supernatant passed through a 0.4-μm sterilizing membrane and lyophilized. B) Extract (250 mg) was suspended in 13 ml of 10 mM sodium phosphate buffer, 0.02% NaN_3, pH 7.0 and centrifuged (30,000 × g, 10 min). 1-ml samples of supernatant were preincubated in water baths at 4, 20, 40, 60, 80, and 100 °C for 5 min and then for a further 5 or 60 min. Samples were dialyzed against water, centrifuged, the supernatants passed through a 0.4-μm sterilizing filter, and lyophilized. Experiments A and B were repeated with extract digested with polyacrylamide-immobilized ribonuclease (Sigma) in 50 mM Tris, 0.02% NaN_3, pH 7.15 for 30 h at room temperature. Following removal of the immobilized enzyme by centrifugation the extracts were treated as before. The A_{280}/A_{260} for the digested extract solution was 1.28 compared with 0.66 before digestion, indicating that new degradation of RNA had indeed occurred. C) Extract (40 mg) was dissolved in 2 ml of 10 mM sodium phosphate buffer, 0.02% NaN_3, pH 7.0. Three other 40-mg samples were dissolved in 2 ml of 10 mM sodium phosphate, 0.02% NaN_3 containing 6 M urea, 8 M guanidine-HCl and 1% Triton X-100 respectively, then adjusted to pH 7.0. All solutions were incubated at room temperature for 3 days, centrifuged and desalted by gel filtration or, in the case of Triton X-100 samples, passed through a 1.0 × 10 cm column of Bio-Beads SM-2 (Bio-Rad) according to Holloway[45]. Before assays, all samples were dialyzed extensively against water and lyophilized.

Extracts of Walker 256 cells were fractionated on CM-Sephadex C50 by suspending the lyophilized powder (12 g) in 100 ml of 0.1 M sodium phosphate buffer, pH 6.1, at 23 °C for 10 min. The suspension was clarified by centrifugation (30,000 × g, 30 min) and the supernatant was dialyzed against the same buffer containing 0.02% azide. After additional centrifugation the supernatant was applied to a 2.5 × 22 cm column of CM-Sephadex C50 equilibrated with the same buffer at a flow rate of 1 ml/min. The column was washed with starting buffer and, when absorbance at 280 nm returned to baseline, with buffer containing 1 M NaCl until absorbance again returned to baseline. The unbound and bound fractions were designated CM-1 and CM-2, respectively. The CM-2 fraction was concentrated 20 times by ultrafiltration with an Amicon PM10 membrane and dialyzed vs 0.05 M sodium phosphate, 0.2 M NaCl, pH 7.0 and applied to a 1.6 × 9 cm column of wheat germ agglutinin-Sepharose previously washed with this buffer. Bound proteins, designated WG-2, were eluted with buffer containing N-acetylglucosamine (100 mg/ml), dialyzed vs water and lyophilized. Several preparations of WG-2 were combined, dissolved in 9 ml 50 mM sodium phosphate, pH 6.0, and chromatographed on Bio-Gel P-100 (2.5 × 100 cm). The fraction not bound to wheat germ agglutinin-Sepharose, WG-1, was concentrated 2–3 times by ultrafiltration (Amicon PM10), dialyzed vs 0.02 M sodium phosphate, pH 6.1, clarified by centrifugation (20,000 × g, 10 min) and applied to a 2.5 × 33 cm column of phenyl-Sepharose. Two peaks of absorbance at 280 nm were resolved by elution with starting buffer and a third was obtained by elution with buffer diluted 1:1 with ethylene glycol. CM-1 was dialyzed vs water, lyophilized, dissolved in 0.05 M Tris-0.15 M NaCl, pH 8.0, clarified by centrifugation and applied to Cibacron Blue-Sepharose in the same buffer. The unbound fraction, CB-1, was dialyzed vs water and lyophilized. The bound fraction, CB-2, was eluted with 0.05 M Tris-0.2 M NaSCN-1 M NaCl, pH 8.0 and dialyzed vs 0.01 M Tris, pH 7.5. It was then applied to a column of DEAE-Sepharose CL-6B equilibrated with the same buffer. The unbound fraction, DE-1, was dialyzed vs. 0.1 M sodium phosphate, pH 7.0 and applied to a column of 5′-AMP-Sepharose-4B in the same buffer. DE-1(AMP) was eluted with buffer containing 1 mM NADH.

Analytical isoelectric focusing was carried out by dissolving lyophilized samples in water, applying them to polyacrylamide slabs containing pH 3.5–9.5 Ampholines and performing analyses with an LKB 2117 Multiphor system according to the manufacturer's instructions. Preparative isoelectric focusing was performed with samples dissolved in water containing 5% pH 5.0–7.0 or 6.0–10.0 LKB Ampholines. Electrophoreses were run in Ultrodex granulated gels and marker prints were made with Whatman No. 1 paper and stained with Coomassie blue. The gels were sectioned with a grid and the pH determined at 1.5-cm intervals with a M1–410 Micro-Combination pH probe (Microelectronics, Inc.). Appropriate sections were combined with two volumes of 0.1 M sodium phosphate, pH 7.0 and poured into plastic syringes fitted with 0.45-μm Millex filters (Millipore Corp.) and washed with buffer. The eluents were dialyzed vs water and lyophilized.

Zinc and copper were determined with a Perkin-Elmer Model 5000 atomic absorption spectrophotometer. Prior to analysis, samples were dissolved in buffer solutions that were freed of adventitious metal contamination by extraction with 0.01% dithizone in carbon tetrachloride or by passage through a Chelex 100 column (Bio-Rad).

4.2 Results

4.2.1 Properties of crude TAF

The male mouse submaxillary gland is one of the few tissues available in quantity that have been cited as potential sources for angiogenesis factors[25]. Hence, our initial attempts to isolate an angiogenesis factor (AF) were carried out with this material. Since this gland is known to contain both nerve growth factor (NGF) and epidermal growth factor (EGF), these two proteins were first isolated using published protocols[73, 85], examined for possible angiogenic activity, and found to be inactive (see discussion). However, other fractions were obtained which indeed induced angiogenesis (CAM me-

thod A), but since gross inflammation was present on a random basis, studies with this tissue were not pursued further.

Our attention then turned to neoplastic tissue which is known to be highly angiogenic and, of course, the only appropriate source of potentially identifiable 'tumor angiogenesis factor' (TAF). Initially we chose to examine the rat Walker 256 carcinoma to define methods for isolation and properties of its angiogenesis factors. This tumor source was selected since it had already served to provide a significant amount of preliminary information (vide infra) and was readily available. Cells grown in culture rather than obtained from tumor bearing animals were employed to assure both a continuous supply and optimum reproducibility of starting material. Moreover, because solid tumors contain high proportions of host matrix and cells, particularly macrophages, this source would unnecessarily complicate TAF purification. Given its presumed mode of action, TAF should be an extracellular molecule and therefore accumulate in the culture medium as it is secreted by the growing cells.

To define the stability of the activity, extracts from rat Walker cells grown in roller bottles were exposed to a wide range of pH, temperature, and denaturing agents prior to testing activity by means of CAM method A and/or rabbit cornea assays. TAF activity was retained after extracts were incubated in solutions of pH 2–10. Pretreatment of the extract by ribonuclease digestion did not affect these stability findings. Attempts to demonstrate susceptibility to proteolytic enzymes were hampered by the fact that small traces of such enzymes themselves induced angiogenesis by causing inflammation. A 5-min incubation at 60 °C decreased activity and at 80 °C destroyed it in both crude and ribonuclease-digested extracts. Extracts treated with urea, guanidine·HCl or Triton X-100 retained their activity after removal of the denaturants by dialysis or chromatography. TAF activity was also stable to treatment with 30%, 50% and 70% ethanol.

Initial fractionation of TAFs was carried out using gel filtration on Sephadex G-100 in 0.05 M Tris-HCl, pH 7.2 as described earlier[27]. Walker cell extracts yielded a material of mol.wt approximately 90,000 that exhibited TAF activity. A rabbit antiserum was raised against it and the resulting immunoglobulin fraction was coupled to Sepharose-6B. This affinity system completely adsorbed the angiogenic activity contained in crude extract. However, the polyspecificity of the antiserum obtained in this fashion precluded substantial purification with respect to the starting material and did not generate sufficient amounts for further fractionation. Nevertheless, it did indicate that the angiogenesis factors in crude extracts are immunogenic.

Preparative isoelectric focusing of Walker cell extracts using a pH 3.5–10 gradient demonstrated the existence of two broad regions of angiogenic activity (CAM method A), one near pH 5 and the other near pH 9. Control ampholines isolated from the same pH regions were inactive on the CAM.

Chromatography of the crude extract on CM-Sephadex C50 in 0.1 M sodium phosphate, pH 6.1 resulted in two active fractions (CAM method A). One did not bind to the column (CM-1) and the other eluted with starting buffer containing 1 M sodium chloride (CM-2) (data not shown). The CM-2 fraction contained 10–20% of the protein applied to the column (table 1), was free of albumin, had an A_{280}/A_{260} of 1.5 compared with 0.7 for the crude extract and contained approximately 2% by weight of sialic acid and neutral sugars. It was also typically more active on the CAM than CM-1.

The complete scheme for purification of TAFs from extracts of Walker 256 cells is depicted in figure 1. Details of the individual steps are described below.

4.2.2. Purification of angiogenic factors from CM-2

Further fractionation of CM-2 was achieved by chromatography on concanavalin A- and wheat germ agglu-

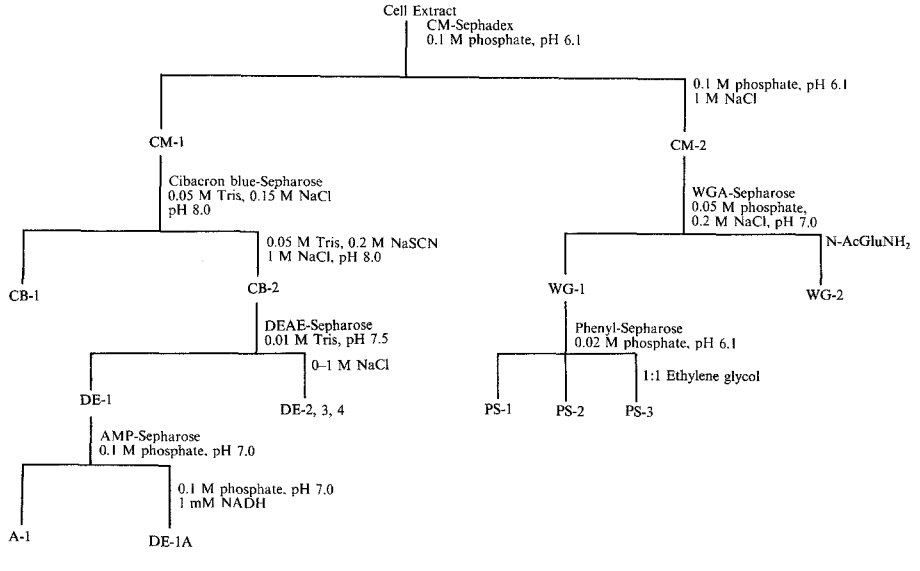

Figure 1. Scheme for the purification of angiogenically active species from extracts of Walker 256 carcinoma cells.

tinin-Sepharose columns. Material binding to Con A-Sepharose and specifically eluting with α-D-methyl-mannoside caused inflammation when assayed on the CAM and was not studied further. However, passage of CM-2 over WGA-Sepharose (fig. 2) yielded a glycoprotein fraction (WG-2) that eluted with N-acetyl-glucosamine and accounted for 2% of the total protein applied to the column (table 1). WG-2 contained 26% hexoses and 7% amino sugars by weight and was active at both 30 and 3 μg when assayed both by CAM method A (table 1) and in the rabbit cornea. This fraction contained one major species of ~ 57,000 daltons plus several minor species as revealed by SDS-PAGE. The low yield of WG-2 rendered further purification efforts difficult. However, preliminary data based on CAM method B indicated that angiogenic activity was included within the column volume on Bio-Gel P-100; had a pI in the range of pH 4–6; was bound to DEAE-Sephacel in 0.1 M Tris-HCl, pH 8.0; and was distinct from the major 57,000 dalton component of WG-2. The CM-2 material which did not bind to WGA-Sepharose (WG-1) was chromatographed on phenyl-Sepharose (fig. 3). Weakly hydrophobic proteins eluted first (PS-1) followed by a more tightly bound peak (PS-2); strongly hydrophobic proteins retained on the column were eluted by adding ethylene glycol to the buffer (PS-3). The three pools contained 30%, 14% and 56% of the Lowry protein respectively, and each had angiogenic activity (table 1). Chromatography of PS-1 on Bio-Gel P-30 (fig. 4) yielded five fractions, two of which (fractions 3 and 4) were active (table 2). Analytical isoelectric focusing of the five fractions showed two major proteins which were common to fractions 3 and 4 but were greatly diminished or not present at all in adjacent fractions. The isoelectric points of these two proteins were approximately pH 6.6 and 9.5, respectively. SDS-PAGE indicated that fractions 3 and 4 contained several proteins of subunit mol.wt ranging from 6,000 to 18,000.

Preparative isoelectric focusing of PS-1 showed that the fraction focusing in the pH range 6.2–6.5 contained all the TAF activity (table 3). Chromatography of PS-1 on DEAE-Sephacel in 20 mM Tris-HCl, pH 8.0 using step elution with NaCl showed TAF activity to be eluted by 20 mM NaCl. The active fraction (table 4) contained several major proteins of subunit mol.wt ranging from 16,000 to 20,000 by SDS-PAGE. In addition, analytical isoelectric focusing indicated that the isoelectric points of all proteins in this fraction range from pH 5.5 to 6.4. Thus, the results of gel filtration, preparative isoelectric focusing and ion-exchange chromatogaraphy all suggest

Table 1. Angiogenic activity from rat Walker carcinoma cell extract

Fraction	Amount (mg)	%	Activity*
Starting material	3632	100	1-2 +
CM-1	1535	42	2 +
CM-2	315	8.7	3 +
WG-1	257	7.1	3 +
WG-2	6	0.2	4/4
PS-1	38	1.0	4/4
PS-2	16	0.4	2/4
PS-3	34	0.9	2/3

*Values followed by + are by CAM method A. Other values are by CAM method B using 50 μg of sample/egg. Responses were recorded at 48 h.

Table 2. Quantitative egg assays of PS-1 fractions obtained by Bio-Gel P-30 chromatography*

Fraction	Amount of sample (μg)		
	200	100	50
1	1/2	1/4	0/3
2	1/1	2/3**	0/4
3	3/3	1/4	2/4
4	2/2	4/4	2/4
5	0/2	1/3	0/3

*Number of positive assays out of total number of eggs using CAM method B. Total number of eggs less than four indicates egg death. Random death of eggs during the course of the assay was due to non-specific causes unrelated to sample. Results were recorded after 48 h. **Inflammation.

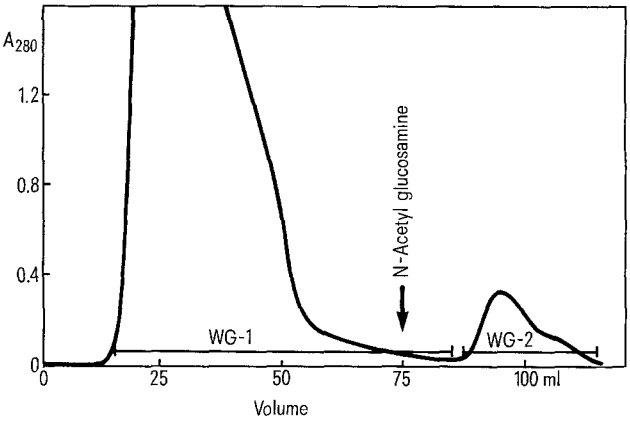

Figure 2. Fractionation of CM-2 on wheat-germ agglutinin-Sepharose. The CM-2 fraction containing 315 mg of protein in 0.05 M sodium phosphate, 0.2 M NaCl, pH 7.0, was applied to a 1.6 × 9 cm column of wheat-germ agglutinin-Sepharose and the column was washed with buffer until the A_{280} of the eluate returned to baseline. Bound proteins were eluted with buffer containing N-acetyl glucosamine, 100 mg/ml (arrow). Fractions designated WG-1 and WG-2 are indicated by the bars.

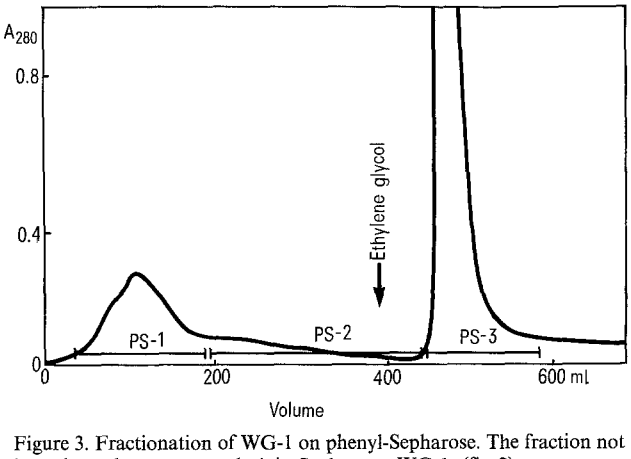

Figure 3. Fractionation of WG-1 on phenyl-Sepharose. The fraction not bound to wheat germ agglutinin-Sepharose, WG-1, (fig. 2) was concentrated by ultrafiltration, dialyzed vs 0.02 M sodium phosphate, pH 6.1, and applied to a 2.5 × 33 cm column of phenyl-Sepharose. The column was eluted with starting buffer until absorbance returned to baseline and bound proteins were then eluted with buffer diluted 1:1 with ethylene glycol (arrow). Fractions designated PS-1, PS-2 and PS-3 are indicated by the bars.

that the angiogenic activity of PS-1 is associated with a protein or proteins of isoelectric point pH 6.0–6.5 and mol.wts of 15,000–20,000.

PS-2 contains one major 33,000 dalton component comprising approximately 50% of the protein in this fraction. This protein was obtained in 90% purity by chromatography on Bio-Gel P-60, but was inactive both on the chick CAM and in the rabbit cornea. Chromatography of PS-2 on DEAE-Sephacel in 20 mM Tris-Cl, pH 8.0 with step elution by NaCl under conditions identical to those used for PS-1, showed activity to be eluted by 50 mM NaCl. As revealed by SDS-PAGE, this fraction, active at 6 μg in the CAM (method B), contains many proteins. Further purification of this activity was hampered by the lack of material, since PS-2 itself represents only 0.4% of the protein present in the crude extract.

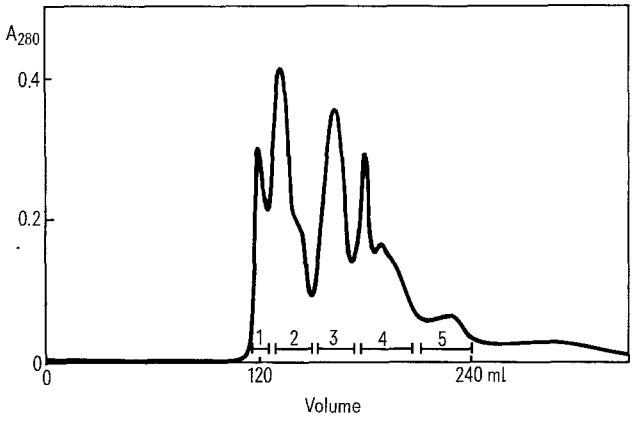

Figure 4. Bio-Gel P-30 chromatography of PS-1. The weakly hydrophobic breakthrough fraction from phenyl-Sepharose chromatography, PS-1 (fig. 3) was concentrated and applied to a 2.5 × 100 cm column of Bio-Gel P-30 equilibrated with 0.05 M sodium phosphate, pH 6.0. The column was eluted with buffer and five pools, indicated by the bars, were obtained.

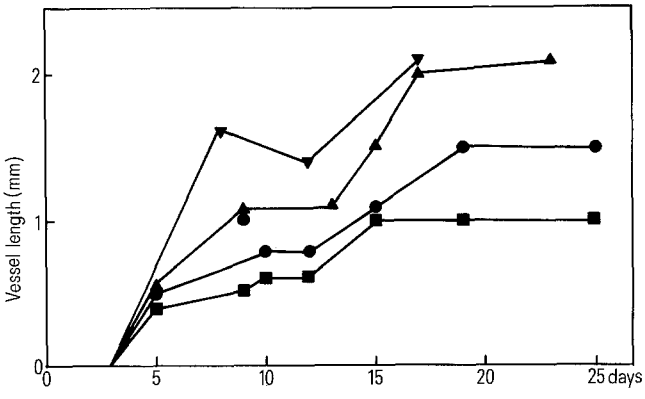

Figure 5. Capillary growth induced by DE-1 implants in rabbit cornea. Lyophilized DE-1, the breakthrough fraction from DEAE-Sepharose chromatography (see text for details of preparation) was suspended in a 10% solution of Elvax 40 in methylene chloride, quickly frozen and dried under vacuum. The amount of DE-1 employed was such that a 1 mm^3 piece of the dried pellet contained either 3 µg (■, ●) or 30 µg (▲, ▼) of protein. A single piece was implanted into a rabbit cornea and blood vessel in growth from the limbus was measured by slit-lamp stereomicroscopy as described[30].

4.2.3 Purification of an angiogenic factor from CM-1

The angiogenically active material which does not bind to CM-Sephadex C50 (i.e. CM-1) was purified by successive chromatography on Cibacron Blue-, DEAE-, and 5'-AMP-Sepharose. Approximately 80% of the CM-1 applied to a column of Cibacron Blue-Sepharose 6B eluted with starting buffer and lacked angiogenic activity. The rest was eluted with 0.05 M Tris-0.2 M NaSCN-1 M NaCl, pH 8.0. This second, angiogenically active fraction, CB-2, was pooled, concentrated, clarified by centrifugation, dialyzed and applied to DEAE-Sepharose CL-6B in 0.01 M Tris, pH 7.5. The unbound fraction, DE-1, was collected, dialyzed against water and lyophilized. It was further purified by adsorption onto 5'-AMP-Sepharose followed by specific elution with 0.5 mM NADH.

Results for angiogenesis assays (CAM method A) during purification of DE-1 are shown in table 5. Substantial activity was usually observed with eggs receiving approximately 50 or more µg of material but little activity was seen with the smaller samples. Using the more quantitative CAM method B, DE-1 preparations were

Table 3. Quantitative egg assays of PS-1 fractions obtained by preparative isoelectric focusing*

Fraction	pH range	Amount of sample (µg)		
		100	50	25
1	4.2–5.3	0/4	0/4	0/4
2	5.4–5.8	0/4	0/4	0/4
3	5.8–6.2	2/4	0/4	0/4
4	6.2–6.5	4/4	3/4	1/4
5	6.6–7.9	0/4	0/4	0/4

*Number of positive assays out of total number of eggs using CAM method B and recorded after 48 h.

Table 4. Quantitative egg assays of PS-1 fractions obtained by DEAE-Sephacel chromatography*

Fraction	Amount of sample (µg)			
	50	25	12	6
Breakthrough	0/4	0/4	0/4	0/4
0.01 M NaCl	0/4	1/4	0/4	0/4
0.02 M NaCl	4/4	2/4	2/4	2/4
0.05 M NaCl	0/4	1/4	1/4	0/4
0.1 M NaCl**	4/4	4/4	4/4	2/4
0.2 M NaCl	2/4	0/4	0/4	0/4

*Number of positive assays out of total number of eggs using CAM method B and recorded at 48 h. **Inflammation present in all assays.

Table 5. Angiogenic activity from rat Walker carcinoma cell extract

Fraction	Amount (mg)	A280	%	CAM activity
Starting material	3792	2442	100	1–2 +
CM-1		2160	88	2 +
Dialysate		1500	61	2 +
CB-1		1350	90	0
CB-2		168	7	2 +
DE-1	4.7	9	0.4	2–3 +
DE-1 (AMP)	4.2	9	0.3	2–3 +

These results are a composite of eight preparations in which a total of 1–2 l of packed Walker 256 cells, obtained from approximately 1000 l of culture medium, were extracted with lactated Ringer's buffer at 4 °C for 4 h to yield 16.7 l of cell extract which was dialyzed vs water and lyophilized to give 3.79 g of starting material. CAM activities were recorded after 48 h using method A.

found to give a positive response in 52% of the eggs when at least 50 µg/egg were tested and in 38% when 25 µg/egg were tested. Below 25 µg/egg most samples were indistinguishable from controls. DE-1 also exhibited angiogenesis activity when assayed in the rabbit cornea using both 3 and 30 µg per implant (fig. 5).

DE-1 could be obtained directly from CM-1 by preparative isoelectric focusing with a pH 6–10 gradient. The fraction focusing between pH 7.7 and 8.5 typically contained the highest angiogenic activity and had properties identical to those of DE-1 prepared by chromatography as described above.

Some information has been obtained regarding the effects of specific chemical modifications on the angiogenic activity of DE-1 (table 6). The protein does not appear to contain a critical, modifiable sulfhydryl group since its activity was unaffected by mercurials or an alkylating agent, nor does it appear to be a serine protease. Activity was abolished by treatment with succinic anhydride but not with acetic anhydride. Blocking arginyl residues with butanedione destroyed angiogenic activity, but tyrosyl residues are either not accessible or not essential, since tetranitromethane and iodine monochloride had little effect.

Inactivation occurred on heating above 60 °C, but treatment with insolubilized ribonuclease or neuraminidase altered neither the activity nor molecular weight of DE-1. Attempts to demonstrate susceptibility to proteolytic enzymes were again hampered by the fact that in our assays small traces of such enzymes induced inflammatory responses that mask true angiogenesis.

4.2.4 Low mol.wt TAF

Purified DE-1 obtained from the AMP-Sepharose column has an isoelectric point in the range of pH 8.5–9.0 but nevertheless it is isolated from the fraction of crude extract that does not bind to CM-Sephadex at pH 6.1. In fact, DE-1 itself does not bind to this resin even after purification to apparent homogeneity. In an effort to understand this unusual chromatographic behavior of DE-1, the protein was dialyzed extensively against 50 mM phosphate buffer, pH 6.1, and subjected to Sephacryl S-200 gel filtration, a procedure that might remove any anionic ligands. Remarkably, two peaks of absorbance at 280 nm were resolved. The first emerged near the void volume of the column, corresponding to the mol.wt of DE-1 while the second was completely included indicating a molecular weight below 5000. Significantly, DE-1 purified by this method now *did* bind to CM-Sephadex at pH 6.1. More importantly, it no longer exhibited any appreciable angiogenic activity either on the CAM or in the rabbit cornea. In contrast, the low mol.wt species elicited a very potent angiogenic response on the CAM, though at least some of this could be attributable to inflammation likely caused by the high concentration of salts in the dried sample.

In order to minimize salt-induced inflammation of the CAM, the low mol.wt fraction from the S-200 column was evaporated to dryness and the residue was extracted with various organic solvents and assayed on the CAM. Methanol was found to be particularly effective. Three out of four eggs tested with methanol extracts were positive after 24 h compared to none for the buffer controls. The UV-absorbing species present in this low mol.wt fraction could not be separated from buffer salts by gel filtration on Bio-Gel P-2 indicating that it had a mol.wt below 1000. The absorption spectrum of both the low mol.wt fraction from the S-200 column and the methanol extract exhibited a maximum at ~ 260 nm and a minimum at ~ 233 nm which were not changed on addition of acid or base.

Since a methanol-soluble species that absorbs maximally at 260 nm could potentially arise from the 5′-AMP-Sepharose column itself or the NADH eluant, methanol extractions were carried out on lyophilized preparations of CM-1 and CB-2. In both cases, the extracts had an absorption maximum at 260 nm and significant activity on the CAM. Neither of them contained copper or zinc detectable by flameless atomic absorption spectroscopy, thus distinguishing them from the low mol.wt copper complexes isolated by McAuslan and coworkers[59, 60].

Methanol extracts of CM-1 were fractionated by ion exchange chromatography on Dowex AG1-X8, by reverse phase HPLC on a C18 column, and by thin layer chromatography on silica gel. Each of these methods revealed the presence of multiple species but none was able to identify the specific molecule responsible for the observed angiogenic activity.

Table 6. Effect of various chemical agents on the angiogenesis activity of DE-1

Reagent	pH	Buffer	CAM activity
None	7.0	Phosphate	2+
$HgCl_2$	7.0	Phosphate	1–2+
N-Ethylmaleimide	7.0	Phosphate	3+
p-Chloromercuriphenylsulfonate	7.0	Phosphate	1+
Phenylmethylsulfonylfluoride	7.0	Phosphate	1–2+
Diisopropylphosphofluoridate	5.0	Acetate	1–2+
Diisopropylphosphofluoridate	6.0	Phosphate	0–1+
Diisopropylphosphofluoridate	7.0	Phosphate	1–2+
Diisopropylphosphofluoridate	8.0	Tris	2+
Succinic anhydride	7.5	Phosphate	0
Iodine monochloride	7.0	Phosphate	1+
Butanedione	8.0	Borate	0
Acetic anhydride	7.5	Phosphate	1+
Tetranitromethane	7.5	Tris	1–2+

4.2.5 Identification of DE-1

Purified DE-1 is devoid of significant angiogenic activity after gel filtration. However, its chemical characteristics were explored because of its possible role as a carrier for the low mol.wt TAF. DE-1 has an isoelectric point in the range of pH 8.5–9.0. Its mol.wt, estimated by electrophoresis in polyacrylamide gradient gels or by gel filtration, is approximately 140,000, whereas by SDS-PAGE it is 35,000, indicating that DE-1 is a tetramer. Its subunit amino acid composition, assuming four identical subunits, is shown in table 5. Based on its ability to bind to both Cibacron Blue- and 5′-AMP-Sepharose as well as the spectral properties of its low mol.wt TAF ligand, DE-1 would appear to have a binding site for nucleotides or structurally related species. A search of the literature revealed that relatively few

known proteins share the isoelectric point, molecular weight, number of subunits, and nucleotide binding characteristics of DE-1. In fact, the only one found to be closely similar was lactate dehydrogenase. Remarkably, the amino acid composition of DE-1 is virtually identical to that of rat LDH-5 (table 7). Enzymatic analysis of DE-1 as well as CM-1 and CB-2 (table 8) confirmed that DE-1 is, indeed, a lactate dehydrogenase. Like DE-1, lactate dehydrogenases from a number of different commercial sources were not angiogenically active on the CAM.

5. *Discussion and summary*

The identification of the molecular messengers that initiate and control the development of complex organ systems is a biological problem of fundamental importance. Blood vessel formation provides an exceptionally suitable means for studying the nature of such chemical factors.

In order to isolate and structurally characterize angiogenic factors (AFs) a number of critical issues need to be resolved. These include: 1) the determination of angiogenic activity by a specific, reliable and valid assay, 2) the definition of a source suitable for the isolation and identification of AFs, and 3) the optimization of the production of AFs from this source to yield material of sufficient quality and quantity to allow chemical characterization.

Table 7. Amino acid composition of DE-1 and rat lactate dehydrogenase[a]

Amino acid	DE-1[b]	DE-1[c]	Rat LDH-5[d]
Asp	35	35	32
Thr	12	12	12
Ser	26	25	24
Glu	32	33	29
Pro	15	14	12
Gly	27	27	24
Ala	20	21	20
Cys[e]	6	6	–
Val	36	34	35
Met	7	7	7
Ile	24	23	22
Leu	39	38	35
Tyr	8	8	7
Phe	7	8	7
Lys	27	28	28
His	6	6	6
Arg	10	10	10

[a] DE-1 results are the average of three analyses and are expressed as residues per subunit based on a subunit mol. wt of 35,000. Hydrolyses were carried out in 6N HCl in vacuum at 110 °C for 20 h. Analyses determined with a Durrum D500 analyzer. [b] Prepared by chromatography as described in the text. [c] Prepared by isoelectric focusing as described in the text. [d] Taken from Goldberg[32] and expressed as residues per subunit. [e] Determined as cysteic acid after performic acid oxidation.

Table 8. Lactate dehydrogenase activity of rat Walker carcinoma cell extract fractions

Fraction	LDH activity (IU/mg)*
CM-1	75
CB-1	0
CB-2	940
DE-1	17,600

* LDH activity was determined by measuring the increase in absorbance at 340 nm on conversion of lactate to pyruvate.

1) Only a limited number of bioassays suitable for the identification of angiogenic activity have been examined in detail. As reviewed in section 2, all such assays involve systems in which tumor cells or extracts are implanted into a living tissue and the growth of new capillaries from the existing vascular bed is observed. The extent of capillary growth is recorded over an interval of several days utilizing stereomicroscopic, histologic or autoradiographic techniques. These methods generally require microgram quantities of material for each assay, and it is often difficult to obtain that amount of sample let alone multiples of it. This clearly precludes checks on reproducibility, precision or accuracy of the method, all of which remain untested. The requirement for delicate surgical manipulation adds another variable of great complexity which can lead to trauma, inflammation and other iatrogenic results.

The assay of Knighton et al.[50] utilizing the chick CAM alleviated many of the shortcomings of earlier methods. This procedure, while useful for initial screening of suitable tumor-derived starting materials and for evaluating preliminary efforts at purification, still suffers from many uncertainties and does not permit critical examination. During the course of these studies, it was replaced by CAM method B which introduced greater reproducibility while using substantially less sample. Nevertheless, neither this nor any of the other procedures available are ideal and do not obviate all concerns. The design and verification of specific, reliable, repeatable and precise methodology to determine angiogenesis remains an imperative of high priority.

2) The choice of an appropriate source containing sufficient material for purification and characterization of AFs is also of prime concern and calls for considerable judgement. Biological messenger molecules are known to be extremely potent and to be present normally in no more than trace quantities. However, under pathological conditions their production may be enhanced substantially. Among normal tissues known to generate hormone-like activities, extracts of adrenal, salivary, and thyroid glands as well as lymph nodes and macrophages have been shown to induce neovascularization[25]. Each of them exhibit known hormonal and stimulatory activity of some type which might make the assignment of angiogenic activity to their specific constituent molecules difficult. Male mouse submaxillary gland, another of this group, at first seemed particularly suitable for identifying molecular species responsible for angiogenic activity, based on its capacity to produce growth factors[73, 85]. We were able to establish that NGF and EGF, two of the known hormone-like growth factors of this gland, are not active angiogenically (CAM method A). EGF was shown subsequently to induce angiogenesis in the cornea[36], but the corneal neovascularization thus induced is accompanied by neutrophil infiltration[31], indicative of inflammation. EGF has no direct effect on capillary endothelium[24], and its lack of activity on the CAM has also been demonstrated elsewhere[66]. Of the submaxillary gland fractions examined that did induce angiogenesis, all generated an intense inflammatory reaction as well, perhaps due to the many proteases

known to be present in these glands[74]. For this reason and because our primary interest was tumor-induced angiogenesis, we decided to abandon this approach.

3) Malignancy continually induces host capillary infiltration of solid tumors[25]. This well-confirmed observation underscored the suitability of tumor-derived material as the most abundant, reproducible source from which to isolate and characterize an angiogenic substance. Although ultimately we were to be concerned with tumors of human origin, we chose initially to examine a rat tumor to define methods for isolation and properties of its AFs. The Walker 256 carcinoma was chosen since prior attempts at purification of TAF employed this source either as a solid tumor or as an established cell line maintained in ascites. Our initial studies confirmed the presence of angiogenic activity in solid tumor homogenates. However, we deemed this source inappropriate as a useful starting material, because solid tumors contain high proportions of host matrix and cells, complicating purification unnecessarily. Subsequently we found that in vitro cultures of Walker 256 cells secrete angiogenic material into the surrounding growth medium[49] as would be expected given the presumed mode of action of TAF as a messenger molecule. Since the growth medium contains 5% FBS and, therefore, large amounts of serum proteins relative to the minute quantities of TAF that might be secreted by the cells, a procedure was developed to extract TAF from the cells by incubating them with serum-free buffer at 4 °C. Although this partially reduced the level of non-TAF protein, nevertheless the yield of crude material from cells maintained in conventional-scale culture, i.e. roller bottles or T-flasks, was a few milligrams per month, hardly adequate for the isolation and characterization of an active species. It was clear that such limitations could only be overcome by producing the tumor cells on a much larger scale. For this purpose, our collaborators at the Monsanto Co. in St. Louis, MO, developed methods and facilities for growing suspension cultures that eventually expanded tumor cell propagation to levels yielding grams of material per week. As might be expected, expansion also amplified many of the difficulties inherent in conventional-scale cell culture procedures. Prominent among these were the choice of growth media to promote rapid cell proliferation and optimal viability; the supply of adequate quantities of high quality, pyrogen-free water; and the elimination of viral, bacterial or fungal contamination. The successful resolution of these issues, through major technical innovations achieved over a period of several years and discussed in detail elsewhere[19, 42a, 81, 82], was critical to our efforts to isolate TAFs.

Moreover, this expansion in quantities of starting materials required the resolution of further issues such as the handling and processing of large volumes of tumor cells and medium, the stability of TAF activity to freezing, lyophilization in the presence and absence of salts, and long and short term storage, etc. For example, gel filtration had already been shown to be an effective first step in the isolation of TAF[27]. The active material from Walker 256 extracts elutes from Sephadex G-100 in a position corresponding to a mol.wt of approximately 90,000. Although this provides an effective initial fractionation, it was impractical for processing the large quantities of extract that had become available. Instead, ion-exchange chromatography was adopted as a first step because of the high capacity of such resins and the fact that analytical isoelectric focusing had identified two well-separated regions of angiogenic activity. Chromatography of conditioned medium on CM-cellulose or CM-Sephadex yielded two active fractions, CM-1 and CM-2, and at the same time concentrated the latter to a level adequate for further purification.

Subsequent processing of CM-2 by wheat germ lectin affinity chromatography, hydrophobic chromatography, isoelectric focusing, ion-exchange chromatography and gel filtration indicated the presence of two major species with angiogenic activity. One, a glycoprotein or glycolipid, binds to wheat-germ agglutinin-Sepharose, is eluted specifically with N-acetylglucosamine and has a pI in the range of 4–6. The second binds to neither the lectin affinity resin nor phenyl-Sepharose, and has the characteristics of a protein of pI 6–6.5 with a mol.wt of 15,000–20,000. Processing of CM-1 yields a low mol.wt angiogenic fraction and a macromolecular component identified as lactate dehydrogenase. Although this latter material is devoid of angiogenic activity, it may function as a carrier. Whether or not this has any physiological significance has yet to be established. The specific angiogenic agents present in CM-1 and CM-2 have not been identified, in part owing to the extremely small quantities of the factors present in extracts of Walker 256 cells, not withstanding the availability of large volumes of such extracts. More significant, perhaps, was our decision to suspend studies of this cell line once conditions were found for growing human tumor cells in large scale suspension cultures. Fortunately, many of the procedures developed with the Walker 256 are directly applicable to human cells. Among the findings to emerge from these studies the most important are that TAFs indeed exist; that they are secreted by tumor cells; that they can, at least in principle, be isolated; and that they are antigenic. This further confirms the TAF hypothesis and points to one possible means for interdicting the process of angiogenesis. While this work was in progress, several other groups reported on angiogenic factors and in some cases reached similar conclusions. The majority of these reports have described low mol.wt substances which in one instance was identified as a copper complex but in all others has not been defined. The low mol.wt TAF found here is not a copper salt but is otherwise uncharacterized. Impure preparations were capable of eliciting a strong angiogenic response on the CAM but any interpretations of this must be made with caution since these samples frequently induced inflammation as well. In addition, while they could be shown to contain many constituents it was not possible to resolve a pure, low mol.wt, angiogenic species. This could be due to low recoveries from the separation procedures employed or to the fact that such molecules are likely to diffuse rapidly on the CAM. The detection of an angiogenic response requires that the active molecule diffuse continually from a focal point so that the characteristic spoke wheel pattern of capillary or venule growth will

be formed. Unless this diffusion persists for 24–48 h the pattern will not occur. It could be that the diffusion of a low mol.wt angiogenesis factor might be retarded in the impure state but not when pure.

It seems unlikely that the angiogenesis activity present in CM-2 is solely due to a low mol.wt species. Treatment with denaturants such as urea, guanidine·HCl or Triton X-100 followed by dialysis or chromatography to remove the agents, did not abolish the activity of the retentate. Also the activity remained associated with a macromolecule through a series of chromatographic steps. In view of the fact that a large number of growth factors are now known to be polypeptides, a protein active as a TAF would not be unreasonable. On the other hand, the active species in CM-1 is most probably of low mol.wt. Interestingly, it appears to be associated primarily with a single type of carrier molecule. However, it is not kown if this interaction occurs in vivo or if it is an artifact arising during the isolation procedure. It is also interesting that this apparent carrier molecule is a lactate dehydrogenase. Much has been written about the relationship of lactate metabolism and neoplasia, and tumor-derived lactate dehydrogenases have been studied in great detail. However, the angiogenesis activity of the low mol.wt material does not require the carrier enzyme, nor is the enzymatic activity of the lactate dehydrogenase affected by the TAF. In addition, lactate dehydrogenase is generally considered to be an intracellular enzyme and would not be expected to be secreted into the culture medium. Its presence therein could reflect cell lysis perhaps during the stationary phase of cell growth. Its association with a low mol.wt angiogenic species might therefore be merely adventitious. Nevertheless, any speculation about the significance of this interaction would be premature in the absence of additional information.

Finally, it would be well to consider some additional pertinent observations. In the past the characterization of known growth factors or hormones that control complex regulatory events has required the resolution of *multicomponent* systems that often involve carrier molecules, activators, receptors, enzyme cascades, and inhibitor-factor complexes. Deducing the organization of such systems necessitates the structural and functional characterization of the individual components. Angiogenesis is, in all probability, no exception.

Neovascularization in vivo is normally under stringent control as evidenced by the lack of vessel proliferation except during embryonic development, post-traumatic tissue restoration and endometrial regeneration[25]. This suggests that critical checks and balances are operating to preclude malfunctions. Hence the initiation, extension and maintenance of the vasculature may well be dependent on multiple molecular species.

At present our efforts are directed to human neoplastic systems. The past experience and approaches reported here have enabled rapid progress in the identification and isolation of human tumor-derived products involved in neovascularization. These findings will be the subject of subsequent reports.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, Dulbecco's calcium- and magnesium free phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AF, angiogenic factor; TAF, tumor-derived angiogenic factor; CAM, chorioallantoic membrane; EGF; epidermal growth factor; NGF, nerve growth factor; LDH, lactate dehydrogenase.

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4 Algire, G.H., An adaptation of the transparent chamber technique to the mouse. J. natn. Cancer Inst. *4* (1943) 1–11.

5 Algire, G.H., and Chalkley, H.W., Vascular reaction of normal and malignant tissues in vivo I. Vascular reactions in mice to wounds and to normal and neoplastic transplants. J. natn. Cancer Inst. *6* (1945) 73–85.

6 Atherton, A., Growth stimulation of endothelial cells by simultaneous culture with sarcoma 180 cells in diffusion chambers. Cancer Res. *37* (1977) 3619–3622.

7 Auerbach, R., Angiogenesis-inducing factors, in: Lymphokines, pp. 69–88. Eds E. Pick and M. Landy. Academic Press, New York 1981.

8 Ausprunk, D.H., and Folkman, J., Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc. Res. *14* (1977) 53–65.

9 Ausprunk, D.H., Falterman, K., and Folkman, J., The sequence of events in the regression of corneal capillaries. Lab. Invest. *38* (1978) 284–294.

10 Baltzer, F., Über metagame Geschlechtsbestimmung und ihre Beziehung zu einigen Problemen der Entwicklungsmechanik und Vererbung (auf Grund von Versuchen an Bonellia). Verh. dt. zool. Ges. *32* (1928) 273–325.

11 BenEzra, D., Mediators of immunological reactions: function as inducers of neovascularisation. Metab. Opthalm. *2* (1978) 2–4.

12 Berman, M., Winthrop, S., Ausprunk, D., Rose, J., Langer, R., and Gage, J., Plasminogen activator (urokinase) causes vascularization of the cornea. Invest. Opthalm. vis. Sci. *22* (1982) 191–199.

13 Brown, R.A., Weiss, J.B., Tomlinson, I.E., Phillips, P., and Kumar, S., Angiogenic factor from synovial fluid resembling that from tumor. Lancet *1* (1980) 682–685.

14 Cavallo, T., Sade, R., Folkman, J., and Cotran, R.S., Ultrastructural autoradiographic studies of the early vasoproliferative response in tumor angiogenesis. Am. J. Path. *70* (1973) 345–362.

15 Chalkley, W.H., Comments on a paper by Algire, G.H., and Legallais, F.Y., Growth and vascularization of transplantable mouse melanomas. Spec. Publ. N.Y. Acad. Sci. *4* (1948) 164.

16 Clark, R.R., Kirby-Smith, H.T., Rex, R.O., and Williams, R.G., Recent modifications in the method of studying living cells and tissues in transparent chambers inserted in the rabbit's ear. Anat. Rec. *47* (1930) 187–211.

17 Cotta-Pereira, G., Sage, H., Bornstein, P., Ross, R., and Schwartz, S., Studies of morphologically atypical ('sprouting') cultures of bovine aortic endothelial cells. Growth characteristics and connective tissue proteins syntheses. J. Cell Physiol. *102* (1980) 183–191.

18 Ehrmann, R.L, and Knoth, M., Choriocarcinoma: transfilter stimulation of vasoproliferation in the hamster cheek pouch-studied by light and electron microscopy. J. natn. Cancer Inst. *41* (1968) 1329–1341.

19 Feder, J., and Tolbert, W.R., The large-scale cultivation of mammalian cells. Scient. Am. *248* (1983) 24–31.

20 Feder, J., Marasa, J.C., and Olander, J.V., The formation of capillary-like tubes by aortic endothelial cells grown in vitro. J. Cell Physiol. *116* (1983) 1–6.

21 Fenselau, A., and Mello, R.J., Growth stimulation of cultured endothelial cells by tumor cell homogenates. Cancer Res. *36* (1976) 3269–3273.

22 Fenselau, A., Watt, S., and Mello, R.J., Tumor angiogenesis factor. Purificaton from Walker 256 rat tumor. J. biol. Chem. *256* (1981) 9605–9611.

23 Folkman, J., Tumor angiogenesis factor. Cancer Res. *34* (1974) 2109–2113.

24 Folkman, J., Angiogenesis; initiation and control, in: Endothelium, pp.212–227. Ed. A.P. Fishman. Ann. N.Y. Acad. Sci. *401* (1982).

25 Folkman, J., and Cotran, R.S., Relation of vascular proliferation to tumor growth. Int. Rev. exp. Path. *16* (1976) 207–248.

26 Folkman, J., and Haudenschild, C.C., Angiogenesis in vitro. Nature *288* (1980) 551–552.

27 Folkman, J., Merler, E., Abernathy, C., and Williams, G., Isolation of a tumor factor responsible for angiogenesis. J. exp. Med. *133* (1971) 275–288.

28 Form, D.M., and Auerbach, R., PGE_2 and angiogenesis. Proc. Soc. exp. Biol. Med. *172* (1983) 214–218.

29 Fournier, G.A., Lutty, G.A., Watt, S., Fenselau, A., and Patz, A., A corneal micropocket assay for angiogenesis in the rat eye. Invest. Ophthalm. vis. Sci. *21* (1981) 351–354.

30 Gimbrone, M.A., Cotran, R.S., Leapman, S., and Folkman, J., Tumor growth and neovascularization: an experimental model using the rabbit cornea. J. natn. Cancer Inst. *52* (1974) 413–427.

31 Goldbaum, M.M., Cleveland, P., Wickham, M.G., and Allen, K., Neovascularization induced by growth factors and by inflammation. Invest. Opthalm. vis. Sci. *19* suppl. (1980) 138 (abstr.).

32 Goldberg, E., Amino acid composition and properties of crystalline lactate dehydrogenase X from mouse testes. J. biol. Chem. *247* (1972) 2044–2048.

33 Goldfarb, R.H., Proteases in tumor invasion and metastasis, in: Tumor cell invasion and metastasis, pp.375–390. Eds L.A. Liotta and I.R. Hart. Martinus Nijhoff, The Hague 1982.

34 Goldman, E., The growth of malignant disease in men and lower animals. Lancet *2* (1907) 1236.

35 Gospodarowicz, D., and Iu, C., Extracellular matrix and control of proliferation of vascular endothelial cells. J. clin. Invest. *69* (1980) 1351–1364.

36 Gospodarowicz, D., Bialecki, H., and Thakral, T.K., The angiogenic activity of the fibroblast and epidermal growth factor. Exp. Eye Res. *28* (1979) 501–414.

37 Gospodarowicz, D., Greenburg, G., Bialecki, H., and Zetter, B.D., Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro *14* (1978) 85–118.

38 Greenblatt, M., and Shubik, P., Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J. natn Cancer Inst. *41* (1968) 111–124.

39 Greene, H.S.N., Heterologous transplantation of mammalian tumors I. The transfer of rabbit tumors to alien species. J. exp. Med. *73* (1941) 461–473.

40 Greene, H.S.N., Heterologous transplantation of mammalian tumors II. The transfer of human tumors to alien species. J. exp. Med. *73* (1941) 475–485.

41 Gross, J.L., Moscatelli, D., and Rifkin, D.B., Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro. Proc. natn. Acad. Sci. USA *80* (1983) 2623–2627.

42 Gullino, P.M., in: Tissue Growth Factors. Handbook of Experimental Pharmacology, vol.57, pp.427–449. Ed. R. Baserga. Springer-Verlag, New York 1981.

42a Harakas, N.K., Lewis, C., Bartram, R.D., Wildi, B.S., and Feder, J., Mammalian cell culture: technology and physiology, in: Eukaryotic Cell Cultures, pp.119–138. Eds R.T. Acton and J.D. Lynn. Plenum Publ. Corp., New York 1984.

43 Hill, C.R., Kussun, R.D., Weiss, J.B., and Garner, A., Angiogenic factor in vitreous from diabetic retinopathy. Experientia *39* (1983) 583–585.

44 Hoffman, H., McAuslan, B., Robertson, D., and Burnett, E., An endothelial growth-stimulating factor from salivary glands. Exp. Cell Res. *102* (1976) 269–275.

45 Holloway, P.W., A simple procedure for removing Triton X-100 from protein samples. Analyt. Biochem. *53* (1973) 304–308.

46 Jakob, W., Jentzsch, K.C., Mauersberger, B., and Heder, G., The chick embryo chorioallantoic membrane as a bioassay for angiogenesis factors: reactions induced by carrier materials. Exp. Path. *15* (1978) 241–249.

47 Karasek, E., and Heder, G., The rabbit cornea model for detecting neovascularizaton effects. Z. VersuchstierKde *23* (1981) 59–66.

48 Keegan, A., Hill, C., Kumar, S., Phillips, P., Schor, A., and Weiss, J., Purified tumor angiogenesis factor enhances proliferation of capillary, but not aortic, endothelial cells in vitro. J. Cell Sci. *55* (1982) 261–276.

49 Klagsbrun, M., Knighton, D., and Folkman, J., Tumor angiogenesis activity in cells grown in tissue culture. Cancer Res. *36* (1976) 110–114.

50 Knighton, D., Ausprunk, D., Tapper, D., and Folkman, J., Avascular and vascular phases of tumor growth in the chick embryo. Br. J. Cancer *35* (1977) 347–356.

51 Kumar, S., Shahabuddin, S., Haboubi, N., West, D., Arnold, F., Reid, H., and Carr, T., Angiogenesis factor from human myocardial infarcts. Lancet *2* (1983) 364–368.

52 Kumar, S., West, D., Daniel, M., Hancock, A., and Carr, J., Human lung tumour cell line adapted to grow in serum-free medium secretes angiogenesis factor. Int. J. Cancer *32* (1983) 461–464.

64 Laemmli, U.K., Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature *227* (1970) 680–685.

54 Langer, R., and Folkman, J., Polymers for the sustained release of proteins and other macromolecules. Nature *263* (1976) 797–800.

55 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein measurement with the Folin phenol reagent. J. biol. Chem. *193* (1951) 265–275.

56 Maciag, J. Kadish, J., Wilkins, L., Stemerman, M.B., and Weinstein, R., Organizational behavior of human umbilical vein endothelial cells. J. Cell Biol. *94* (1982) 511–520.

57 Madri, J.A., Williams, S.K., Wyatt, T., and Mezzio, C., Capillary endothelial cell cultures: phenotypic modulation by matrix components. J. Cell Biol. *97* (1983) 153–165.

58 Makarski, J.S. Jr, Evidence for reversible morphologic phenotypes (sprouts) of cultured endothelial cells. Cell Biol. int. Rep. *6* (1982) 225–233.

59 McAuslan, B.R., A new theory of neovascularization based on identification of an angiogenic factor and its effect on cultured endothelial cells, in: Control mechanisms in animal cells, pp.285–292. Eds L. Jimenez de Asua, R. Shields, R. Levi-Montalcini and S. Iacobelli. Raven Press, New York 1980.

60 McAuslan, B.R., and Hofman, H., Endothelial stimulating factor from Walker carcinoma cells. Relation to tumor angiogenic factor. Exp. Cell Res. *119* (1979) 181–190.

61 McKenzie, H.A., pH and buffers, in: Data for biochemical research, p.485. Eds M.C. Dawson et al. University Press, Oxford 1969.

62 Muthukkaruppan, V.R., and Auerbach, R., Angiogenesis in the mouse cornea. Science *205* (1979) 1416–1418.

63 Muthukkaruppan, V.R., Kubai, L., and Auerbach, R., Tumor-induced neovascularization in the mouse eye. J. natn. Cancer Inst. *69* (1982) 699–708.

64 Needham, J., New advances in the chemistry and histology of organized growth. Proc. R. Soz. Med. *29* (1936) 1577.

65 Nicosia, R.F., Tchao, R., and Leighton, J., Histiotypic angiogenesis in vitro: light microscopic, ultrastructural, and radioautographic studies. In Vitro *18* (1982) 538–549.

66 Phillips, P., and Kumar, S., Tumor angiogenesis factor (TAF) and its neutralisation by a xenogenic antiserum. Int. J. Cancer *23* (1979) 82–88.

67 Phillips, P.J., Steward, J.K., and Kumar, S., Tumor angiogenesis factor (TAF) in human and animal tumours. Int. J. Cancer *17* (1976) 549–558.

68 Polverini, P.J., Cotran, R.S., Gimbrone, M.A., and Unanue, E.R., Activated macrophages induce vascular proliferation. Nature *269* (1977) 804–806.

69 Raju, K.S., Alessandri, G., Ziche, M., and Gullino, P., Ceruloplasmin, copper ions, and angiogenesis. J. natn. Cancer Inst. *69* (1982) 1183–1188.

70 Rosenthal, A.R., Appleton, B., and Hopkins, J.L., Intraocular copper foreign bodies. Am. J. Opthalm. *78* (1974) 671–677.

71 Sanders, A.G., and Shubik, P., A transparent window for use in the Syrian hamster. Israel J. exp. Med. *11* (1964) 118.

72 Sandison, J.C., A new method for microscopic study of living growing tissues by the introduction of transparent chamber in the rabbit's ear. Anat. Rec. *28* (1924) 281–287.

73 Savage, C.R. Jr, and Cohen, S., Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. J. biol. Chem. *247* (1972) 7609–7611.
74 Schenkein, I., Levy, M., Franklin, E.C., and Fragnione, B., Proteolytic activity of mouse submaxillary gland. Archs. Biochem. Biophys. *182* (1977) 64–70.
75 Schor, A.M., Schor, S.L., and Kumar, S., Importance of a collagen substratum for stimulation of capillary endothelial cell proliferation by tumour angiogenesis factor. Int. J. Cancer *24* (1979) 225–234.
76 Selye, H., On the mechanism through which hydrocortisone affects the resistance of tissues to injury. J. Am. med. Ass. *152* (1953) 1207–1215.
77 Shubik, P., Vascularization of tumors: a review. J. Cancer Res. clin. Oncol. *103* (1982) 211–226.
78 Spemann, H., Neue Arbeiten über Organisatoren in der tierischen Entwicklung. Naturwissenschaften *15* (1927) 947.
79 Stockley, A.T., The chorioallantoic membrane of the embryonic chick as an assay for angiogenic factors. Br. J. Dermat. *102* (1980) 738.
80 Suddith, R.L., Kelley, P.J., Hutchison, H.T., Murray, E.A., and Haber, B., In vitro demonstration of an endothelial proliferative factor produced by neural cell lines. Science *190* (1975) 682–684.
81 Tolbert, W.R., and Feder, J., Large-scale cell culture technology, in: Annual Reports on Fermentation Processes, vol. 6. Eds G.T. Tsao and M.C. Flickinger. Academic Press, New York (in press).
82 Tolbert, W.R., Schoenfeld, R.A., Lewis, C., and Feder, J., Large-scale mammalian cell culture: design and use of an economical batch suspension system. Biotech. Bioengng *24* (1982) 1671–1679.
83 Tsukamoto, K., and Sugino, Y., Tumor angiogenesis activity in clonal cells transformed by bovine adenovirus type 3. Cancer Res. 39 (1979) 1305–1309.
84 Tuan, D., Smitz, S., Folkman, J., and Merler, E., Isolation of the nonhistone proteins of rat Walker carcinoma 256. Their association with tumor angiogenesis. Biochemistry *12* (1973) 3159–3165.
85 Varon, S., Nomura, J., and Shooter, E.M., Reversible dissociation of mouse nerve growth factor protein into different subunits. Biochemistry *7* (1968) 1296–1303.
86 Virchow, R., Die krankhaften Geschwulste. Hirschwald, Berlin 1863.
87 Weiss, J.B., Brown, R.A., Kumar, S., and Phillips, P., An angiogenic factor isolated from tumours: A potent low-molecular-weight compound. Br. J. Cancer *40* (1979) 493–496.
88 Willis, R.A., The Borderland of Embryology and Pathology. Butterworth and Co., London 1958.
89 Zetter, B.R., Migration of capillary endothelial cells is stimulated by tumor-derived factors. Nature *285* (1980) 41–43.
90 Zetter, B.R., The endothelial cells of large and small blood vessels. Diabetes *30*, suppl. 2 (1981) 24–28.
91 Ziche, M., Jones, J., and Gullino, P.M., Role of prostaglandin E_1 and copper in angiogenesis. J. natn. Cancer Inst. *69* (1982) 475–482.

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Mini Reviews

Superparasitism reconsidered: is it an adaptive competition? The example of *Diadromus pulchellus*

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Key words. Superparasitism; endoparasites; *Diadromus pulchellus;* adaptive competition.

With superparasitism, several larvae of solitary entomophagous endoparasites can be found in the same host, and yet only one of them develops. The distribution of eggs can range from uniform distribution to an apparently random distribution or to a contagious distribution, which generates superparasitism.

Diadromus pulchellus WSM. is an Ichneumonid solitary endoparasite of *Acrolepiopsis assectella* pupae. Analysis of its egg distribution is possible because egg-laying is followed by withdrawal of the ovipositor. The size of eggs permits their precise count in host pupae. With 5 hosts of the same age, the females conspicuously concentrate their eggs in a limited number of hosts (table 1). An analysis of the distribution of the eggs reveals a statistically significant concentration compared to a random distribution[1,2]. Likewise, field parasitization by *D. pulchellus* shows a tendency to a contagious distribution. But this evidence is indirect because the hosts are not dissected: on 90 collected hosts, Rousse[3] found 36 stung pupae with on average 2.78 stings per pupa. The same type of distribution has been observed for example in *Pleolophus basizonus*[4]. It is very surprising that females do not avoid superparasitism in spite of their complex sensorial equipment[3] and that wasps constantly probe the pupa's surface with their antennae. The females reject hosts affected by virosis, and systematically select the younger hosts[1]. The presence of

Table 1. Parasitized, superparasitized hosts and fecundity of *D. pulchellus* in experiments conducted in the years 1977 and 1980

	Number of experiments	Available hosts N	Eggs laid	Parasitized hosts n	n/N	Superparasitized hosts n′	n′/n
1977	950	4750	2797	1846	0.39	666	0.36
1980	836	4180	2840	2034	0.48	806	0.28