Reports

Oxygen Tension Regulates the Expression of Angiogenesis Factor by Macrophages

Abstract. *When cultured in a hypoxic environment similar to that found in the center of a wound, macrophages secreted active angiogenesis factor into the medium. Under conditions similar to those of well-oxygenated tissue, macrophages did not secrete active angiogenesis factor. Macrophages that secreted the factor at hypoxic conditions stopped secreting it when returned to room air. Thus the control of angiogenesis in wound healing may be the result of macrophages responding to tissue oxygen tension without the necessity of interacting with other cell types or biochemical signals.*

The ease with which mammals initiate and control the healing of wounds belies the complexity of this phenomenon. A wound, whether traumatic or surgical, initiates an acute inflammatory response and activates coagulation, fibrinolysis, the immune system, and the mononuclear phagocyte system. After the debridement of damaged tissue at the wound site, the extracellular matrix and its microvasculature are reestablished. Unlike other physiological processes, normal wound healing has an abrupt initiation and a regulated termination (*1*).

Macrophages are essential for normal wound repair (*2*). As scavengers, they phagocytose foreign particles and microorganisms. Proteolytic enzymes released by macrophages aid in debridement (*3*). The requirement for macrophages in the processes contributing to angiogenesis and tissue regeneration in the wound is well established (*4, 5*), but the regulatory signals to which they respond are not fully known. This study provides evidence that macrophage-directed angiogenesis is not only initiated in response to the reduction of environmental oxygen, but is also terminated when environmental levels of oxygen are reestablished.

Adherent macrophages derived from rabbit bone marrow were cultured for 14 days (*6*) on gas-permeable 60 by 15 mm Permanox culture dishes (Lux Scientific). Gas-permeable dishes were used because cells cultured on normal tissue culture dishes would become hypoxic under 3 to 5 mm of medium regardless of the ambient O_2 concentration. All experiments were performed in serum-free Dulbecco's modified Eagle's medium supplemented with 0.2 percent lactalbumin hydrolysate.

When macrophages were cultured under normal tissue culture conditions they were placed in an incubator with 10 percent CO_2 and air. When cultured under hypoxic conditions, the culture dishes were placed in sealed culture chambers containing 10 percent CO_2, 0 to 10 percent O_2, and the balance N_2. After the cells had been cultured for 24 hours at a low O_2 concentration, the medium was passed through a 0.45-μm filter to remove any detached cells and then dialyzed against 0.01*N* acetic acid or 20 m*M* ammonium bicarbonate in a dialysis bag with a molecular weight cutoff of 2000. The retentate was lyophilized and reconstituted to give a tenfold concentrate. A typical experiment involved 2×10^6 macrophages maintained in 12 ml of medium at each O_2 concentration.

To study the expression of macrophage angiogenesis factor after recovery from hypoxia, medium was collected after 24 hours of culture at 2 percent O_2. The cells were then washed and recultured for 24 hours at 20 percent O_2. Angiogenesis was determined by the corneal implant assay (*7*). Mitogenic activity was determined by measuring the incorporation of [^{3}H]thymidine by serum-starved rabbit brain capillary endothelial cells or primary fibroblasts (*8*).

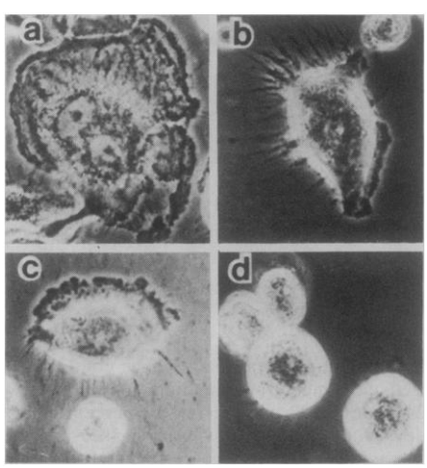

Fig. 1. Morphological response of macrophages cultured at various oxygen concentrations. Rabbit bone marrow macrophages were cultured in gas-permeable culture dishes at 20 percent oxygen for 14 days and then for 24 hours at 20 percent (a), 5 percent (b), 2 percent (c), or 0 percent (d) oxygen (magnification, ×780).

Macrophages exposed to various O_2 concentrations for 24 hours showed an O_2-dependent change in morphology (Fig. 1). Typically, bone marrow macrophages cultured in 20 percent O_2 (equivalent to room air) were adherent and well spread. Some of the cells exhibited a bipolar morphology that is characteristic of bone marrow macrophages. When cultured under increasingly hypoxic conditions the cells became increasingly rounded. In 2 percent O_2 the macrophages were spherical and began to detach.

To determine whether macrophages secrete a biologically active angiogenesis factor, cell-free medium was recovered from macrophages grown at O_2 concentrations of 0 to 20 percent. The corneas implanted with pellets of concentrated medium conditioned by macrophages grown at 20 percent O_2 (Fig. 2, a, j, and k) were indistinguishable from corneas implanted with control Hydron pellets, that is, they were clear, avascular, and noninflamed. The angiogenic activity of medium conditioned by macrophages grown at 10 percent O_2 (Fig. 2b) and at 5 percent O_2 (Fig. 2c) was 0.25 U and 0.5 U, respectively. However, medium conditioned by macrophages grown at 2 percent O_2 was markedly angiogenic, having 1.5 U of angiogenic activity (Fig. 2, d, l, and m). The new blood vessels were dense and well developed in the normally avascular cornea. Macrophages grown at 0 percent O_2 (Fig. 2e) secreted less angiogenesis factor into the medium (1.0 U) than cells grown at 2 percent O_2 (Fig. 2d).

When 1×10^7 live rabbit bone marrow macrophages were injected into rabbit corneas, an angiogenic response of 1.5 U was detected; no angiogenic response resulted from the injection of 1×10^6 cells (Table 1) (*9*). In histological sections of corneas implanted with 1×10^7 macrophages (Fig. 2, h and i), the implantation caused a thickening of the cornea owing to an increase in the number of fibroblasts and the amount of collagen in the corneal stroma. The sections also verified that capillaries migrate into the normally avascular cornea. These observations confirm that live

Table 1. Angiogenic and mitogenic activity of live macrophages and macrophage-conditioned medium; N.D., not done.

Source of activity	Angiogenic activity per cornea (U) (7)	Mitogenic activity (percent of activity in control cultures with 10 percent serum)	
		Fibroblasts*	Rabbit brain capillary endothelial cells†
Live macrophages			
1×10^6	0	N.D.	N.D.
5×10^6	0.5	N.D.	N.D.
1×10^7	1.5	N.D.	N.D.
Conditioned medium‡			
2×10^4, 20 percent O_2, 24 hours	0	19	34
2×10^4, 2 percent O_2, 24 hours	1.5	17	32
2×10^4, 2 percent O_2, 24 hours; then 20 percent O_2, 24 hours§	0	19	30

*Fibroblasts were isolated from outgrowths of explants of adult rabbit skin (17). †These cells were isolated and cultured by a modification (8) of a method for isolating rat brain capillary endothelial cells (18). ‡Medium was conditioned by macrophages maintained at the indicated oxygen concentration for the time indicated, then dialyzed and concentrated as described in text. §Medium was discarded after the first 24 hours, the cells were washed, medium was replaced, and activity was assayed after the second 24 hours.

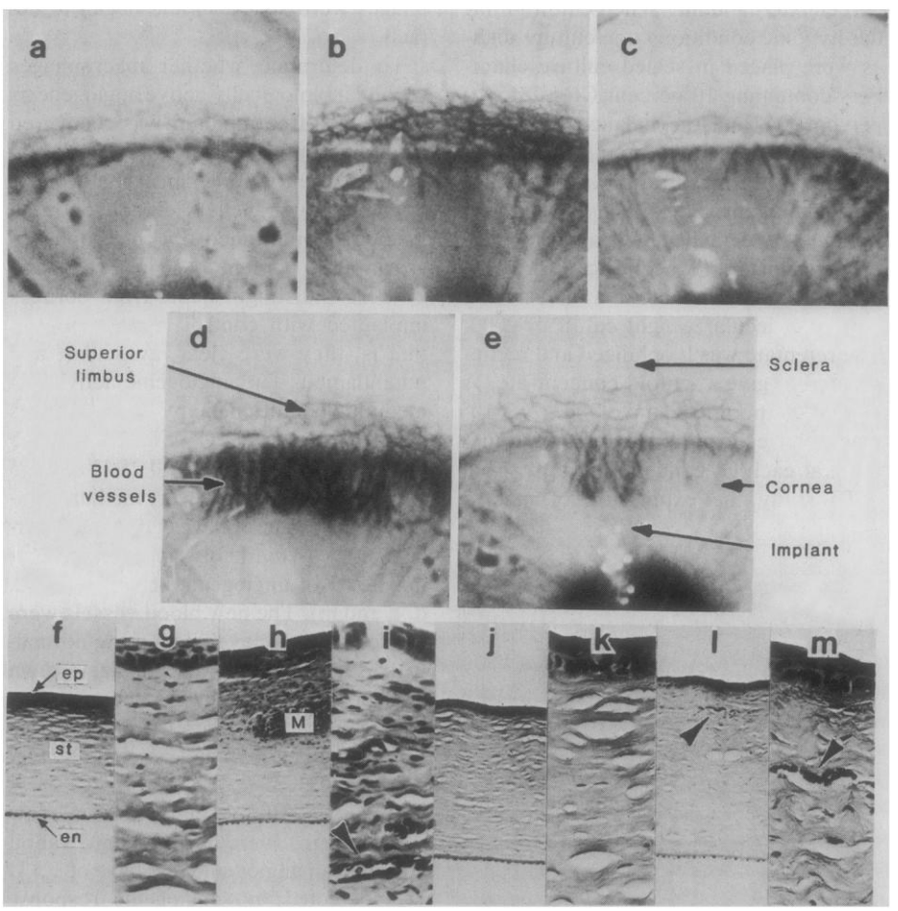

Fig. 2. Angiogenic response resulting from the implantation of macrophage-conditioned medium in rabbit corneas. Implants were of dialyzed and concentrated medium conditioned by rabbit bone marrow macrophages cultured for 24 hours at 20 percent (a), 10 percent (b), 5 percent (c), 2 percent (d), or 0 percent (e) O_2. New blood vessels growing from the superior limbus are indicated in (d). Orientation of all panels is the same. Panels (c), (d), and (e) show typical angiogenic responses that would be scored as 0.5, 1.5, and 1.0 U of activity, respectively (7). (f to m) Histological sections of control cornea (f and g), corneas implanted with 1×10^7 rabbit marrow macrophages (h and i), and corneas implanted with Hydron pellets containing the equivalent of the medium conditioned by 2×10^4 macrophages cultured at 20 percent O_2 (j and k) and 2 percent O_2 (l and m). In (f) the corneal epithelium (*ep*), stroma (*st*), and endothelium (*en*) are indicated. The edge of the macrophage implant (*M*) is indicated in (h). Blood vessels containing red blood cells are indicated by the arrowheads in (i), (l), and (m). Magnifications: ×76 (f, h, j, and l) and ×200 (g, i, k, and m).

macrophages stimulate angiogenesis from the corneal limbus and mitogenesis in the corneal stroma.

Although 1×10^7 live macrophages produced 1.5 U of angiogenic activity, medium conditioned for 24 hours by only 2×10^4 macrophages cultured at 2 percent O_2 produced the same amount of activity (Table 1). In histological sections, corneas implanted with medium conditioned by macrophages grown at 2 or 20 percent O_2 (Fig. 2, j to m) did not show evidence of the stromal thickening or infiltration of inflammatory cells characteristic of the implantation of live macrophages (Fig. 2, h and i). Capillary infiltration was seen only in the corneas implanted with medium from hypoxic macrophages.

We tested the macrophage-conditioned media for their ability to stimulate mitogenic activity, as measured by [^{3}H]thymidine uptake by fibroblasts and rabbit brain capillary endothelial cells (Table 1). The secretion of macrophage mitogens was not increased by hypoxic growth conditions and is, therefore, regulated independently of angiogenesis factor. The isolation of a nonmitogenic angiogenesis factor from rabbit wound fluid (8) established that angiogenesis and mitogenesis are not identical.

Because wound hypoxia is a transient condition eventually replaced by normal tissue O_2 concentration during the final stages of healing, we tested the effect of recovery from hypoxia on the secretion of angiogenesis factor by macrophages. On return to normal O_2 conditions (20 percent O_2), the first indication of recovery from hypoxia was the resumption (within 30 minutes) of a well-spread morphology typical of cells that had never been exposed to hypoxic conditions. The medium conditioned by macrophages grown at 2 percent O_2 for 24 hours was angiogenic, but the medium for these same cells was not angiogenic once the cells were returned to normal O_2 conditions for 24 hours (Table 1). These data establish that tissue O_2 concentration can act as both a positive and negative regulator of the angiogenic potential of macrophages.

Because the vasculature has been destroyed by the injury, the central portion of a wound becomes hypoxic (10). Experimental elimination of the O_2 gradient extending from the hypoxic center to the oxygenated edge of the wound halts angiogenesis (11). The partial pressure of O_2 (PO_2) in the tissue of an individual breathing atmospheric air at sea level varies from 30 to 60 mmHg, depending on the degree of vascularization (10).

The PO_2 of the dead space of a soft tissue wound varies from 20 mmHg to near-anoxia. Our data show that less than 2×10^4 macrophages cultured under conditions similar to those of a wound (2 percent O_2, 15 mmHg) and (0 percent O_2, near-anoxia) actively stimulate angiogenesis. When cultured at a PO_2 similar to that of arterial blood (10 percent O_2, 76 mmHg) and to that of tissue (5 percent O_2, 38 mmHg), macrophages did not stimulate a substantial angiogenic response. Under normal tissue culture conditions (20 percent O_2, 152 mmHg) in gas-permeable culture vessels, there was no angiogenic response at all. However, normal tissue culture O_2 concentrations are unlikely to be experienced by normal tissues other than alveoli.

The relation of angiogenesis to tissue hypoxia has been observed in other systems. The central core of a malignant neoplasm is hypoxic (*12*) owing to the metabolic hyperactivity of tumor cells and the lack of circulation in the central portion of the tumor. Macrophages constitute 10 to 30 percent of the cells in mouse (*13*) and human (*14*) tumors, but the significance of this is controversial (*15*); no correlation has been found between macrophage content of tumors and tumor vascularization. However, because the secreted products of fewer than 2×10^4 macrophages stimulate angiogenesis under hypoxic conditions, it is possible that macrophages caught in the growing tumor will experience hypoxia sufficient to increase the amount of active macrophage angiogenesis factor. The similarities, if any, between tumor angiogenesis factor (*16*) and macrophage angiogenesis factor are not known. Nevertheless, macrophages may contribute to the vascularization of certain rapidly growing hypoxic tumors.

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9. That the implantation of 10^7 and not 10^6 whole macrophages was necessary to stimulate angiogenesis may also be explained as hypoxic regulation. The PO_2 of the corneal stroma has been measured at as low as 30 mmHg [M. Kwan, J. Niinikoski, T. K. Hunt, *Invest. Opthalmol.* **11**, 108 (1972)]. When 10^7 metabolically active cells are implanted into the stroma, they may rapidly deplete the stroma of O_2 and establish sufficient hypoxia to stimulate the expression of angiogenesis factor.
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Cell Surface P-Glycoprotein Associated with Multidrug Resistance in Mammalian Cell Lines

Abstract. *The plasma membranes of hamster, mouse, and human tumor cell lines that display multiple resistance to drugs were examined by gel electrophoresis and immunoblotting. In every case, increased expression of a 170,000-dalton surface antigen was found to be correlated with multidrug resistance. This membrane component is of identical molecular size and shares some immunogenic homology with the previously characterized P-glycoprotein of colchicine-resistant Chinese hamster ovary cells. This finding may have application to cancer therapy.*

Selection of variants in mammalian cells that are resistant to specific drugs, such as *Vinca* alkaloids, maytansine, colchicine, anthracyclines, actinomycin D, or bleomycin, is often accompanied by expression of a complex phenotype of cross resistance to various unrelated drugs (*1–14*). This characteristic is referred to as the multidrug resistance phenotype. The generation of such variants in tumor cells may be an important mechanism by which neoplasms become resistant to treatment by combination chemotherapy.

Studies in model systems indicate that multidrug resistance results from a reduced cellular accumulation of the drugs involved (*5–19*), and changes in the plasma membrane have been observed (*17–23*). In the well-characterized colchicine-resistant (CH^R) Chinese hamster ovary (CHO) system, for example, genetic analyses involving cell-cell hybrids, drug-sensitive revertants, and DNA-mediated transformants of the CH^R phenotype indicate that multidrug resistance, colchicine resistance, and reduced drug accumulation are the result of the same genetic alteration (*21, 24, 25*). Moreover, the expression of a 170,000-dalton plasma membrane glycoprotein (P-glycoprotein) is invariably associated with this pleiotropic phenotype (*20–22, 24*). The degree of drug resistance is correlated approximately with the amount of P-glycoprotein present (*20, 22*). The objective of the present study is to determine whether or not P-glycoprotein expression is also associated with the multidrug resistance phenotype observed in other cell systems.

Each of the different mammalian cell lines examined in this study (Table 1) was originally selected for resistance to a specific drug, and in each case a multidrug resistance phenotype typified by cross resistance to unrelated compounds was observed. Such a phenotype appears to reflect a membrane-associated alteration (*6, 24, 26*). We therefore pre-