PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **172**, 214–218 (1983)

PGE_2 and Angiogenesis[1] (41548)

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Abstract. The angiogenic capability of PGE_2 was tested by implanting pellets of an ethylene vinyl acetate slow release polymer containing PGE_2 on the chorioallantoic membrane of 8-day-old chicken embryos. Elvax pellets releasing approximately 0.2, 2.0, or 20 ng/day PGE_2 were found to induce neovascular responses. In contrast, pellets releasing 2.0 or 20 ng/day of either PGA_2, PGF_2, or TXB_2 did not appear to be angiogenic when compared with PGE_2. These release rates of PGE_2 are similar to those reported for a variety of tumors, activated macrophages, inflammatory exudates, and rheumatoid synovia, suggesting that PGE_2 may be a key factor in various neovascular reactions.

New blood vessel formation, angiogenesis, is essential for the successful growth of solid tumors (1, 2). Angiogenesis can be induced not only by tumor cells, but also by allogeneic lymphocytes (3, 4) and by activated macrophages (5). In fact, angiogenesis-inducing factors have been obtained from a large number of tissue sources (6), suggesting that the induction of blood vessels may be critical for a number of biological processes ranging from wound healing to cancer growth, and including vascular reactions associated with inflammation and autoimmune pathology.

The idea that prostaglandins of the E series might play a significant role in many of the angiogenesis-inducing systems has been proposed (6, 7), although experimental data in support of the concept have been quite limited (8). Reports of the association of PGE_2 with tumors (10), activated macrophages (11–13), and inflammatory exudates (14) lend credence to this idea and have prompted us to carry out experiments to examine whether prostaglandin E_2 can induce angiogenesis.

Materials and Methods. The assay system we have employed is the assessment of vascular response on the chlorioallantoic membrane (CAM) of chicken embryos grown in petri dish embryo culture (15). In brief, embryos were explanted from 3-day eggs into 100 × 20-mm plastic tissue culture dishes (Falcon). To provide adequate humidity, each dish was placed in a larger (150 × 25-mm) plastic dish (Falcon) and incubated at 37°. On Day 8, implantation was performed by making an incision in the CAM with a 23-gauge needle and placing the implant over the hole. With sufficient care, bleeding was minimal. This method has the advantage of permitting the monitoring of the neovascular reactions including sequential photography and measurement of the blood vessels surrounding the angiogenesis-stimulating implant.

We used Elvax-40 (ethylene vinyl acetate copolymer, DuPont Chemical Co.) as a slow-release compound into which test samples of prostaglandin (Sigma Chemical Co.) could be introduced (16). The method used was a modification of that used by Rhine *et al.* (16). Elvax was washed at least 50 times in 2.5% ethanol at 37° (at least 3 hr per wash) before use. Washed Elvax was dissolved in methylene chloride (10% w/v) at 37° in siliconized glass tubes. The appropriate prostaglandin, in an ethanol carrier, was added and the mixture was vortexed and poured into glass molds on dry ice. The solidified mixture was dried at −20° for 2 days, and for a further 2 days under mild vacuum at room temperature. This technique resulted in Elvax slabs of consistent dimensions which could be cut into uniform implants.

Disc-shaped pellets, 2 mm diameter, each containing approximately 1 mg of polymer were used as implants on the CAM of 8-day-old chicken embryos. Controls for uni-

[1] This research supported by NIH Grants EY-3243, AI-14607, and CA-28626.

[2] David M. Form is a postdoctoral trainee supported by National Cancer Institute Training Grant CA-09106.

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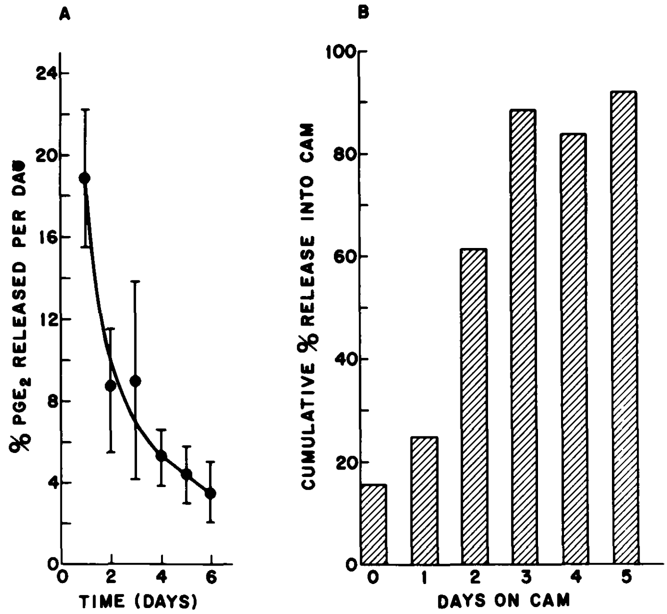

FIG. 1. Release of [^{3}H]PGE_2 from Elvax pellets. (A) Percentage released per day into saline. (B) Cumulative percentage released into the CAM. Calculated from the amount of [^{3}H]PGE_2 remaining in pellets after implantation on the CAM for various periods of time (21).

formity and release kinetics were carried out by using [^{3}H]PGE_2 (prostaglandin E_2 [5,6,8,11,12,14,15-^{3}H(N)], New England Nuclear). Pellets containing [^{3}H]PGE_2 were placed individually in glass scintillation vials containing 1 ml saline. At 24-hr intervals, the pellets were transferred to fresh scintillation vials. After 6 days, the experiment was terminated and the vials were dried and assayed for radioactivity. The release of PGE_2 into the CAM was calculated from the amount of [^{3}H]PGE_2 remaining in pellets after implantation on the CAM for various periods of time.

Elvax pellets were prepared containing PGE_2 or other cyclooxygenase products (PGE_1, PGA_2, PGF_2, thromboxane B_2 [TxB_2]). Control pellets consisted of Elvax alone. In order to insure a more even release rate, pellets were soaked in saline for 24 hr before use.

For each experiment, 8-day-old embryos were divided into groups of six, each group receiving only one type of pellet. Two pellets were implanted on each CAM, resulting in 12 pellets per group. All embryo cultures were subsequently coded to prevent potential bias and each pellet area was scored daily for extent of neovascular response. The scoring method used was a modification of that used by Folkman (1). Pellets received a score of (0) if negative, (+) if weakly positive, and (+2) if clearly positive. At the end of the experimental period, the embryos were uncoded and a coefficient of angiogenesis (COA) was calculated for each group by dividing the total observed score for all pellets by the maximum possible score.

For statistical analysis, the data were subjected to a one-way analysis of variance followed by the Newman–Keuls *t* test for comparison between groups.

Results. As shown in Fig. 1A, [^{3}H]PGE_2 is released gradually from pellets over a 6-day period. Release rates for concentrations ranging over three logs were found to be comparable from 10^{-2} to 20^{-5} mg, and conformed to the published release rates of Rhine *et al.* (16) for release of bovine serum albumin. As can be seen in Fig. 1A, the initial release was rapid, but a steady slow rate was obtained after the first day, and for this reason, all pellets used for testing angiogenesis were held in

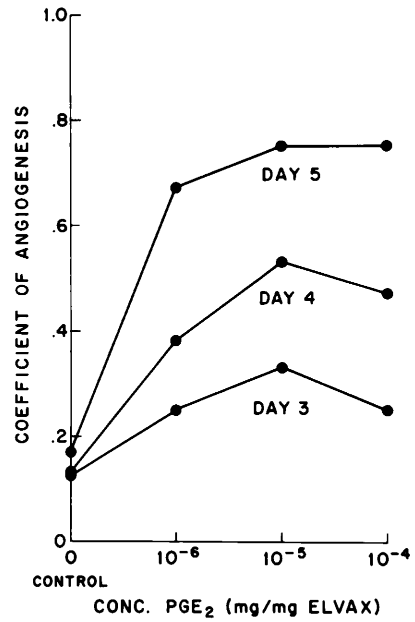

FIG. 2. Coefficient of angiogenesis (COA) calculated for Elvax pellets containing 10^{-6}, 10^{-5}, or 10^{-4} PGE_2. COA calculated 3, 4, and 5 days after implantation of the pellets on the CAM of 8-day-old chicken embryos. Values shown were obtained from the median of four experiments (21).

saline for 24 hr before being placed on the CAM for assessment of angiogenesis-inducing activity. The release rates of PGE_2 into the CAM during a 5-day period is shown in Fig. 1B.

The response of the CAM vasculature to the presence of PGE_2-containing pellets involved two distinct phenomena: (1) the formation of a network of dilated capillaries occurred within 48 hr and declined after 96 hr; and (2) the appearance of bending, looping, and sprouting of blood vessels, with a clear orientation toward the implanted pellet. This reaction was seen beginning on the third day and became increasingly marked over the next several days.

Pellets containing 10^{-4}, 10^{-5}, or 10^{-6} mg PGE_2 releasing approximately 0.2, 2.0, or 20 ng/day into the CAM, were tested for their angiogenesis-inducing capacity. The median values of four experiments are shown in Fig. 2. By Day 5, the COA values for all three concentrations of PGE_2 were significantly higher ($P < 0.025$) than controls. PGE_1 gave results similar to those seen with PGE_2 (data not shown).

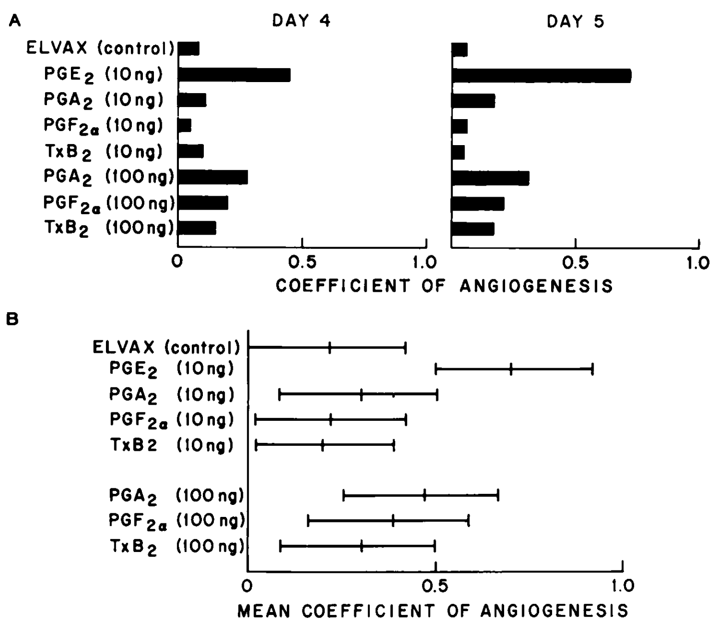

FIG. 3. Comparison of the angiogenesis capacity of PGE_2 with that of other prostaglandins. (A) Coefficient of angiogenesis (COA) calculated for Elvax pellets containing 10^{-6} mg PGE_2 compared to pellets containing 10^{-5} or 10^{-6} mg PGA_2, PGF_2, or TxB_2. COA calculated 4 and 5 days after implantation of the pellets on the CAM of 8-day-old chicken embryos. (B) Mean COA per egg on Day 5 ± one pooled standard deviation (95% confidence interval).

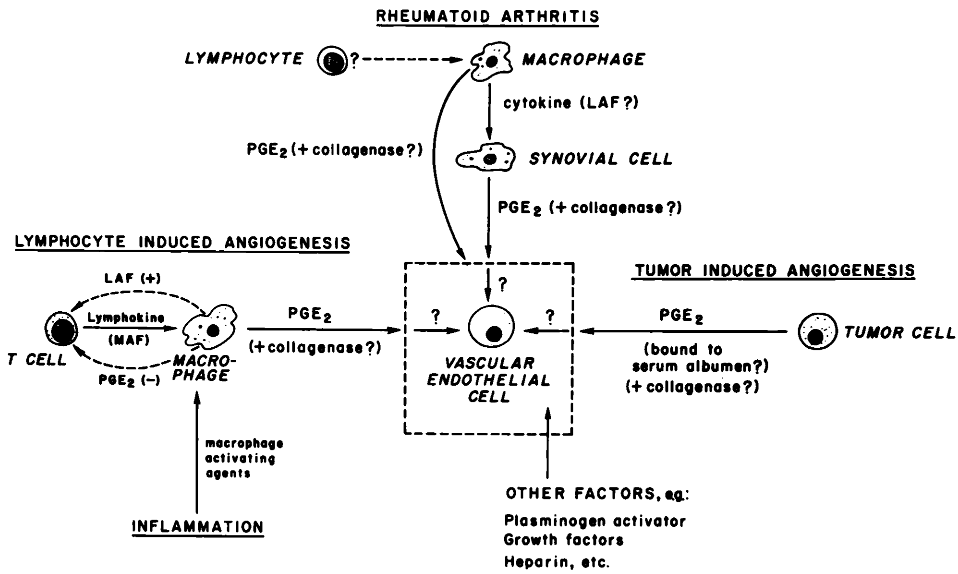

FIG. 4. Possible points of PGE involvement in angiogenesis (see text for discussion).

As a further control for specificity, the angiogenic capability of pellets containing 10^{-4} or 10^{-5} mg PGA_2 (the oxidation product of PGE_2), $PGF_{2\alpha}$, or TxB_2 were compared with pellets containing 10^{-5} mg PGE_2. The results of a typical experiment are shown in Fig. 3. Only PGE_2 is clearly angiogenic.

Discussion. The fact that PGE_2 can be angiogenic in our test system at release rates of as little as 0.2 ng/day induced us to reexamine data on neovascularization induced in a variety of other experimental or clinical situations to determine if the levels of PGE_2 are consistent with its being involved in angiogenesis. Indeed, levels of PGE_2 at or above those used by us have been reported for a variety of tumors (10), for activated macrophages (11–13), and for inflammatory exudates (14), all of which have been associated with angiogenesis. Neovascularization is also found associated with a proliferating vasculature in the synovium, and it is of interest not only that both cultured rheumatoid synovial fragments and adherent cells derived from such fragments produce PGE_2 at high rates (17), but that PGE_2 production can be further enhanced by supernatants from activated macrophages (18).

A major group of neovascular reactions appears to be initiated by antigen-stimulated lymphocytes (lymphocyte-induced angiogenesis, LIA) (6), and is seen in graft-versus-host reactions, autoimmune reactions, and delayed hypersensitivity. Although lymphocytes themselves produce little or no PGE, it may be significant that LIA almost definitely involves release of soluble factors (lymphokines) (3, 4, 19, 20) and there is ample evidence that macrophages activated by lymphokines produce high levels of PGE_2 (11–13).

Biochemical studies of angiogenesis-inducing factors have pointed to two classes of factors: low-molecular-weight ones (200–450 daltons), and larger, nondialyzable ones, some of which may actually involve a small active component attached to a larger carrier molecule (6). Whether the active molecule associated with these factors will prove to be PGE_2 is not clear, but there is a strong possibility that molecules of the prostaglandin–leukotriene family may be involved. Figure 4 summarizes some of the hypothetical points at which PGE_2 could play a role in angiogenesis, and its relationship to other factors.

There is no reason to expect that these small molecules should be the only mediators of neovascularization, for angiogenesis is a complex biological process, which can be controlled at many points. Dissolution of vascular continuity, induction of cell migration, triggering of endothelial cell mitosis, induction of capillary sprouts, extension of vascular continuity, and reestablishment of blood flow must all occur in an orderly fashion. On the other hand, the fact that PGE$_2$ in amounts equivalent to those seen in inflammation, in synovial fluid, in leukocytic infiltrates, and in tumor nodules can by itself elicit angiogenesis suggests that the array of factors capable of inducing neovascularization may become reduced to a few key substances.

We wish to thank David Rhude for his expertise and assistance during the formative stages of this work, and Dr. Younan Sidky for helpful discussions and suggestions.

1. Folkman J. Tumor angiogenesis. In: Becker FF, ed. Cancer. New York, Plenum Press, Vol. 3:p355, 1976.
2. Folkman J, Cotran R. Relation of vascular proliferation to tumor growth. Int Rev Exp Pathol **16**:207–248, 1976.
3. Sidky YA, Auerbach R. Lymphocyte-induced angiogenesis (LIA): A quantitative and sensitive assay of the graft vs. host reaction. J Exp Med **141**:1084–1108, 1975.
4. Auerbach R, Sidky YA. Nature of the stimulus leading to lymphocyte-induced angiogenesis. J Immunol **123**:751–754, 1979.
5. Polverini PJ, Cotran RS, Gimbrone MA, Jr, Unanue ER. Activated macrophages induce vascular proliferation. Nature (London) **269**:804–806, 1977.
6. Auerbach, R. Angiogenesis-inducing factors: A review. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p69, 1981.
7. Ben Ezra D. Neovasculogenesis. Triggering factors and possible mechanisms. Surv Ophthalmol **24**:167–176, 1978.
8. Ben Ezra D. Neovasculogenic ability of prostaglandins, growth factors and synthetic chemoattractants. Amer J Ophthalmol **86**:455–461, 1978.
9. Cooper CA, Bergamini MVW, Leopold IH. Use of flurbiprofen to inhibit corneal neovascularization. Arch Ophthalmol **98**:1102–1105, 1980.
10. Owen K, Gomolka D, Droller MJ. Production of prostaglandin E$_2$ by tumor cells *in vitro*. Cancer Res **40**:3167–3171, 1980.
11. Stenson WI, Parker CW. Prostaglandins, macrophages and immunity. J Immunol **125**:1–5, 1980.
12. Gemsa D. Stimulation of prostaglandin E release from macrophages and possible role in the immune response. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p355, 1981.
13. Morley J. Role of prostaglandins secreted by macrophages in the inflammatory process. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p377, 1981.
14. Vane JR. Prostaglandins as mediators of inflammation. Advan Prostaglandin Thromboxane Res **2**:791–801, 1976.
15. Auerbach R, Kubai L, Knighton D, Folkman J. A simple procedure for the long-term cultivation of chicken embryos. Dev Biol **41**:391–394, 1974.
16. Rhine WD, Hsieh DS, Langer R. Polymers for sustained macromolecule release: Procedures to fabricate reproducible delivery systems and control release kinetics. J Pharm Sci **69**:265–270, 1980.
17. Dayer JM, Krane SM, Russell GG, Robinson DR. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. Proc Nat Acad Sci (USA) **13**:945–949, 1976.
18. Krane SM, Dayer JM. A mononuclear cell factor modulates collagenase and PGE$_2$ production by rheumatoid synovial cells. In: De Weck AL, Krisensen F, Landy M, eds. Biochemical Characterization of Lymphokines. New York, Academic Press, p439, 1980.
19. Sidky YA, Auerbach R. Response of the host vascular system to immunocompetent lymphocytes: Effects of preimmunization of donor or host animals. Proc Soc Exp Biol Med **161**:174–177, 1979.
20. Roehm NR, Sidky YA, Auerbach R. Lyt phenotype analysis of the effector cells responsible for evoking lymphocyte-induced angiogenesis. Cell Immunol **63**:272–278, 1981.
21. Form DM, Sidky YA, Kubai L, Auerbach R. PGE$_2$-induced angiogenesis. Prostaglandins and Cancer: First International Conference. New York, Alan R. Liss, p685, 1982.

Received June 7, 1982. P.S.E.B.M. 1983, Vol. 172.