Enhancement of FGF-like polypeptides in the retinae of newborn mice exposed to hyperoxia

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Retinae from neonatal mice exposed to prolonged hyperoxia (100% oxygen), show marked vasoproliferation Extracts of such retinae were chromatographed on a heparin-Sepharose column and the absorbed material subjected to HPLC fractionation. Two components, approx. 10 and 18 kDa, respectively, were found to have angiogenic activity, which was higher than in corresponding extracts from animals exposed to air. Both fractions and an additional 5 kDa component reacted with an antibody to basic fibroblast growth factor (bFGF) and showed higher levels in hyperoxia The data suggest that hyperoxia activates angiogenic factors belonging to the heparin binding family

Angiogenesis; Fibroblast growth factor; Hyperoxia; Polypeptide; Retina

1. INTRODUCTION

Angiogenesis, the outgrowth of new blood capillaries, is a fundamental process in the development of tissues and organs during embryogenesis, tissue regeneration and wound healing [1]. It involves several complex phenomena, the resolution of the basal membrane, migration and proliferation of endothelial cells which develop vessel sprouts that eventually form capillary loops [2]. Abnormal angiogenesis can be pronounced in some pathological states, such as rheumatoid arthritis, cancer and certain retinopathies (proliferative diabetic retinopathy, retinal vein thrombosis and retinopathy of prematurity [3]).

Angiogenesis can also be induced in the eyes of newborn animals, including mice, by exposure to hyperoxia during the first week of life [4]. This was confirmed in the present paper which describes an attempt to recover and characterize angiogenic factors from retinae of hyperoxic mice. Chromatographic fractions were tested for angiogenic activity in the chick embryo chorioallantoic membrane. The fractions were also tested for immunogenic basic fibroblast growth factor (bFGF) which is a heparin binding growth factor, with mitogenic activity on endothelial cells and with angiogenic activity [5,6]. Originally isolated from nervous tissue [7], bFGF is known to be produced in several tissues, among those the retina [5]. Basic FGF is a polypeptide of an apparent molecular mass of about 18 kDa [8].

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2. MATERIALS AND METHODS

2.1. *Polypeptides and chemicals*

Recombinant bFGF (*E. coli*) was purchased from Boehringer-Mannheim. Protein and peptide standards used for column calibration were from KabiVitrum AB, Stockholm, Sweden (human growth hormone) and from Sigma. The chromatographic material, heparin-Sepharose and Sephadex G-25 (PD-10), were obtained from Pharmacia, Uppsala, Sweden. Rat monoclonal anti-bFGF antibodies were a gift from Dr Andrew Baird, Dept of La Jolla, CA, USA. All other chemicals and solvents were of analytical grade.

2.2. *Preparation of animals*

Litters of 10–15 newborn NMRI albino mice (Alab, Stockholm, Sweden), younger than 15 h were exposed to 100% oxygen for 5 days and then kept in air for 10 more days. Newborn mice maintained in air for 15 days served as controls. At the end of the 15-day period animals of both groups were anaesthetized with a lethal dose of mebumal injected intraperitoneally and perfused with ice-cold PBS (phosphate-buffered saline) from the left heart ventricle. The eyes were enucleated and the retinae removed without delay and immediately frozen and stored at −70°C until further processing. Some animals were, subsequent to the PBS perfusion, injected with India ink from the left heart ventricle in order to visualize the ocular vessels [4].

2.3. *Purification procedures*

The tissues were homogenized in 1 M acetic acid (10 ml/g tissue) and heated to 90°C for 15 min. Following cooling on ice and centrifugation (20 min at 10000 × *g*) the extract was collected and directly applied onto a 2 ml column of heparin-Sepharose, previously equilibrated in 1 M acetic acid. After sample application the column was washed with 10 ml of 1 M acetic acid and eluted with 2.5 ml 3 M NaCl. The eluate was subsequently desalted on a prepacked Sephadex G-25 column (PD-10) with 1 M acetic acid as eluent. The polypeptide-containing fraction was lyophilized and redissolved in 200–300 μl of 20 mM Tris-HCl, pH 7.4. The reconstituted material was further purified by HPLC-gel filtration. In these experiments a Pharmacia/LKB instrument equipped with an Ultropac TSK G-3000 Sw column (7.5 × 600 mm) was used. This column operated at a flow

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rate of 0.5 ml/min and fractions of 0.5 ml were collected and lyophilized for further analysis.

2.4. *Assay for angiogenic activity*

Peptide-containing fractions obtained from the HPLC-purification were tested for angiogenic activity on the chick embryo chorioallantoic membrane (CAM). The CAMs of 8-day-old explanted chick embryos were used [9]. Peptide-containing and control fractions (collected in glass tubes) were thoroughly mixed with Elvax, an ethylene/vinyl acetate copolymer prepared according to Langer and Folkman [10], dissolved in dichloromethane and dried to pellets, 3 mm in diameter. The pellets were then placed on the CAM of the explanted chicken embryos. The vessel architecture around the pellet was evaluated 5 days later and quantified according to the method given by Vu et al. [11]. In brief, the angiogenic response was scored according to a 5 grade scale, e g. grade 0 = no angiogenic response, to grade 4 = very pronounced 'spoke-wheel' effect on the vessels converging to the pellet. The ratio between the observed grade of angiogenesis and the maximum attainable score designated the angiogenic coefficient is thus a figure between 0 and 1

2.5. *Radioimmunoassay*

The radioimmunoassay (RIA) for bFGF used antibodies raised in rabbits against human $bFGF_{1-24}$ which are specific for the FGF_{10-20} sequence [12]. Their crossreactivity with acidic FGF is less than 1%. ^{125}I-labelled bFGF was used as tracer. All incubations were performed in Eppendorf tubes at 4°C and lasted for 48 h. The antibodies were diluted (1:10000) in 10 mM Na-phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.82% NaCl and 0 93% EDTA (RIA-buffer). Lyophilized HPLC-separated samples were redissolved in 500 μl methanol/0.1 M HCl (1:1). The reaction mixture contained a 25 μl aliquot of sample or standard, 100 μl antiserum dilution and 100 μl labelled peptide (5000–6000 cpm in RIA-buffer). All incubations were performed in Eppendorf tubes at 4°C and lasted for 48 h. Following incubation, 100 μl calf serum (Gibco, Scotland) and subsequently 1 ml 15% polyethylene glycol 6000 were added to each vial. The samples were left to stand at 4°C for 30 min before centrifugation in a Beckman Microfuge B (2 min at 10000 rpm). The supernatants were decanted and the pellets taken for counting in a gamma-counter. The detection limit of the radioimmunoassay was 500 fmol/tube and 50% of tracer binding was observed at 50 fmol/tube. The coefficient of interassay variation was 8.4%.

3. RESULTS

The choice of acidic pH for the extraction of the angiogenic polypeptides was based on several experiments, in which different pHs were tried. Extraction at neutral pH resulted in large amounts of peptides and proteins with affinity for the heparin-Sepharose gel (results not shown). The acetic acid extraction combined with the affinity chromatography resulted in 2 or 3 small UV-absorbing, presumably peptide components particularly in the oxygen-exposed animals, separable by HPLC gel filtration (Fig. 1). The peptides in the fractions 31–32 and 35–36, respectively corresponding to the highest peaks in Fig. 1 (oxygen-exposed animals), showed an angiogenic coefficient of 0.5 in the CAM assay, while the corresponding fractions from the control animals showed an angiogenic coefficient of 0.25. From calibration runs with peptide and protein standards the apparent molecular masses of these fractions were estimated to be approximately 18 and 10 kDa, respectively. The 18 kDa fraction

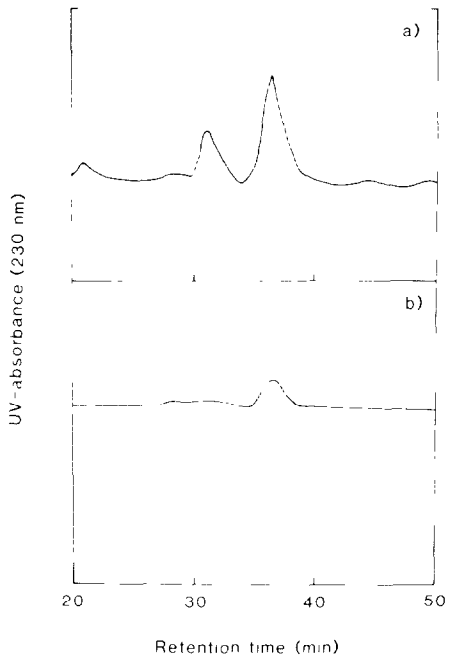

Fig. 1. UV-absorbance following HPLC-gel filtration of mouse retinal extracts bound to the heparin-Sepharose column. The retinae material was collected from mice exposed to (a) oxygen and (b) air. The HPLC column (TSK-G3000 SW) was eluted with 20 mM Tris-HCl, pH 7.8, maintaining a flow rate of 0.5 ml/min

chromatographed almost identically with bFGF standard. In the radioimmunoassay for bFGF both fractions were active. A third component eluted at about 5 kDa molecular mass (Fig. 2).

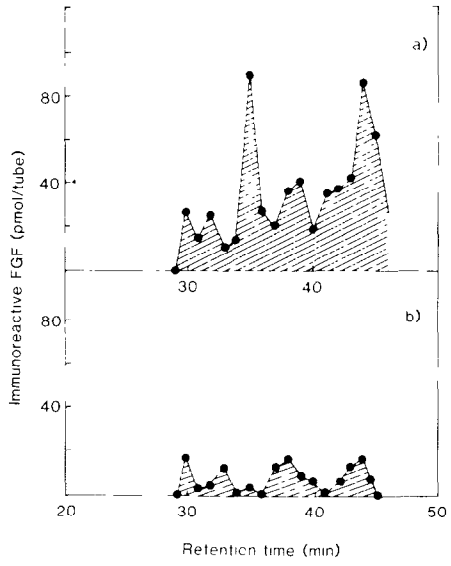

Fig. 2 The distribution of bFGF-like immunoreactivity following HPLC-gel filtration (TSK-G3000 SW) of the heparin-Sepharose adsorbed fraction from mouse retina collected during exposure to (a) oxygen and (b) air.

4. DISCUSSION

Ashton [4] has described the vasoproliferative effects of hyperoxic treatment on the retinal vessels in newborn animals of several species, including mice. The same vascular effects of hyperoxia could be reproduced in the present study (not shown). Michaelson [13] has proposed 'a factor or factors' that regulate the normal development of the normal vascularization of the retina, and later Ashton and coworkers [14] suggested a 'vasoformative' factor inducing the retinal neovascularization not only in experimental situations but also in conditions with proliferative retinopathies. Since FGF has been detected in the retina [15], this growth factor may be one of those postulated. Our results show that the increased proliferation induced by hyperoxia is accompanied by an increase in a heparin binding angiogenic factor and an increased production of polypeptides reacting in a radioimmunoassay for bFGF. More work is needed to establish whether the biologic activity is indeed due to a bFGF-like molecule. Further studies are also needed to establish the time relationship between exposure to hyperoxia, the vascular proliferation and the increased production of angiogenic polypeptides.

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