Increased procollagenase activating angiogenic factor in the vitreous humour of oxygen treated kittens

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SUMMARY Previous studies have demonstrated an increase in a low molecular weight angiogenic factor (ESAF) present in the retinæ of kittens with oxygen induced retinopathy. The present paper describes differences in the quantity of ESAF extracted from the vitreous humour of control and oxygen treated animals and proposes a mechanism for the induction of intravitreal neovascularisation.

The existence of an angiogenic factor in retinal tissue undergoing active vascularisation was first proposed by Michaelson in 1948. It was suggested that such a factor would be a small freely diffusible molecule, the release of which could be brought about by hypoxia. Release of such a factor into the vitreous humour and its accumulation therein would bring about the vascularisation of this tissue by retinal vessels.

Retinal extracts have been shown not only to promote angiogenesis in the cornea and the chick chorioallantoic membrane but also to contain a low molecular weight endothelial cell angiogenic factor (ESAF). The quantitative assay of this molecule can be achieved by virtue of its ability to activate skin fibroblast procollagenase in a dose dependent manner, and differences in the quantities of ESAF present in normal and oxygen treated retinæ have already been described. Other recent studies have shown ESAF to be present in vitreous humour taken from both normal eyes and from those with proliferative diabetic retinopathy. Hence it appears that in conditions of proliferative retinal vascular growth there occur quantitative changes in the ESAF normally present in the retina and vitreous humour, rather than de novo synthesis or release of a disease-specific angiogenic molecule from the retina.

The absence of intravitreal neovascularisation in the normal eye may be ascribed to endogenous inhibitors of angiogenesis, which are present in sufficient quantities to inhibit any neovascularisation induced by endogenous angiogenic molecules. One of these vitreal inhibitors has been shown to inhibit collagenase and hence acts in a manner opposing the procollagenase activating action of ESAF. In pathological conditions, however, sufficient angiogenic molecules may diffuse from the retina to overcome the natural avascularity of the tissue.

In an earlier report we showed that destruction of the vasculature of the retina, such as to render it hypoxic, gives rise to elevated levels of ESAF within the retina. We now present evidence that this factor is diffusible and passes into the vitreous, where it is the presumed stimulus to the subsequent preretinal angiogenesis.

Materials and methods

Newborn kittens were incubated for a period of three days in an atmosphere containing 70–80% oxygen. They were then removed abruptly to normal room air for a period of between nine and 18 days before administration of an excess of pentobarbital and vitrectomy under deep anaesthesia. Normal healthy kittens not exposed to hyperoxia were used as controls.

Vitreous humour from each individual kitten was homogenised for 15 seconds at 4°C in an equal volume of 50 mM NH4 HCO3 buffer (pH 7.9) containing 2 M MgCl2 and was clarified by
Increased procollagenase activating angiogenic factor
centrifugation at 20000 g (1 h, 4°C). The supernatant
was assayed for protein\(^\text{13}\) prior to ultrafiltration on a
YM5 filter membrane (5000 Mr exclusion limit)
(Amicon Ltd, Stonehouse, Gloucs) with five volumes
of bicarbonate buffer.
The ultrafiltrate was reduced by rotary evaporation
to a volume of 5 ml and applied to an octadeccyl
silica column (Analyticchem, Harbor City, Califor-nia). Bound low molecular weight angiogenic
material was eluted with methanol.
Assay of angiogenic material for its ability to
activate procollagenase was performed according to
the method of Weiss et al.\(^\text{9}\) Results were expressed as
µg collagen degraded/h/mg protein in the super-
natant.

Results

Extracts of vitreous humour from both control and
oxygen treated kittens contained significant quan-
tities of a low molecular mass angiogenic factor.
Reverse phase chromatography permitted the iso-
lation of a purified fraction which when applied to a P2
column eluted in a position consistent with ESAF.\(^*\)
This fraction was assayed for its ability to
activate procollagenase. From the results (Table 1) it is
apparent that vitreous humour from oxygen treated
kittens contains significantly increased quantities

Table 1  Activation of procollagenase* by ESAF\(^*\) present in
the vitreous humour of control and oxygen treated kittens

<table>
<thead>
<tr>
<th>Experiment</th>
<th>µg Collagen degraded/h/mg protein in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxygen treated</td>
</tr>
<tr>
<td>1</td>
<td>2.00</td>
</tr>
<tr>
<td>2</td>
<td>1.26</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>2.25</td>
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<tr>
<td>6</td>
<td>1.61</td>
</tr>
<tr>
<td>7</td>
<td>1.04</td>
</tr>
<tr>
<td>8</td>
<td>0.91</td>
</tr>
<tr>
<td>9</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean</td>
<td>1.25</td>
</tr>
<tr>
<td>SD</td>
<td>0.59</td>
</tr>
<tr>
<td>p=0.01</td>
<td></td>
</tr>
<tr>
<td>Mean retinal results from Taylor et al.(^9)</td>
<td>2.24</td>
</tr>
</tbody>
</table>

Paired vitreous humours from individual kittens were used in each
experiment.
*The procollagenase used in the assay had very little intrinsic
collagenolytic activity; enzyme without ESAF degraded less than
0.35 µg collagen per hour.
*ESAF itself in the absence of procollagenase had no effect on the
collagen substrate.
SD = standard deviation.

of low molecular weight procollagenase activating
material as compared to controls.
Variation within the two groups is likely to reflect
individual differences between animals, though a
temporal relationship between duration of exposure
to a normal atmosphere and the quantities of ESAF
present may exist in the oxygen treated group.
These results complement our earlier study of the
effects of hypoxia on the angiogenic potential of the
developing kitten retina.\(^9\) The differences between
the vitreous humour samples from the oxygen treated
and control kittens were smaller than those found in
the retinal extracts.

Although the quantities of ESAF in the control
vitreous humour and retinae (expressed as µg colla-
gen degraded/h/mg protein) were similar, in the
oxygen treated kittens the retinae contained approxi-
mately twice as much ESAF as comparable samples
of vitreous humour. Hence an imbalance existed
between the quantities of ESAF present in the
retinæ and vitreous humours of oxygen treated
kittens.

Discussion

The concept of tissue hypoxia as a stimulus to
angiogenesis is not new,\(^14\)-\(^17\) and a hypoxic gradient
has been shown to be mandatory for wound healing
angiogenesis.\(^18\)
There is also evidence that a similar situation
pertain in respect of the retina, but it was largely
cumustential until we demonstrated that artificially
induced retinal ischaemia is followed by raised tissue
levels of a specific angiogenic fraction (ESAF).\(^19\) This
provides an explanation for the revascularisation of
the retina which occurs in newborn kittens subjected
to vaso-obliterrative doses of oxygen. However, such
animals also develop preretinal new vessels, and our
findings in the present report support the view that
this reflects diffusion of the initially intraretinal factor
into the vitreous. It appears, moreover, that the
promotion of new vessel growth is a function of
opposing influences, since there is, even in control
animals, a basal level of ESAF (0.50 µg collagen
degraded/h/mg protein). The presence of antiangi-
genic substance(s) in the vitreous of other species is
well established.\(^11\)-\(^19\) The level of ESAF in the
vitreous compares with that found in the retina of
the same animals (0.59 µg collagen degraded/h/mg
protein), which suggests that there is little obstacle
to passage of the factor across the inner limiting
membrane of the retina. It may, in passing, be noted
that the presence of significant quantities of ESAF in
the retina is likely to be a reflection of the normal
processes of developmental vascularisation occurring
in the neonate kitten at birth. When the level of
ESAF rises in the retina in response to ischaemia, this creates a diffusion gradient and an increase in vitreal ESAF concentration which may be sufficient to overwhelm the opposing angiogenic inhibitory substances and permit extraretinal neovascularisation.

The sequence of reduced intraretinal circulation, tissue hypoxia, release of ESAF with diffusion into the vitreous, and subsequent preretal angiogenesis is not confined to the artificially induced situation in kittens but is likely to be relevant in the context of naturally occurring proliferative retinopathy in man. The presence of angiogenic factor in the vitreous has already been observed in respect of diabetic retinopathy and certain other non-diabetic conditions associated with preretal angiogenesis (Elstow SF, et al. in preparation), and its origin from an inadequately perfused retina is entirely consistent with angiographic findings.

References

Accepted for publication 27 November 1986.
Increased procollagenase activating angiogenic factor in the vitreous humour of oxygen treated kittens.

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*Br J Ophthalmol* 1988 72: 2-4
doi: 10.1136/bjo.72.1.2

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