Quantitation of angiogenesis factor in bovine retina and tumour extracts by means of radioimmunoassay

S. SHAHABUDDIN AND S. KUMAR

From the Christie Hospital, Manchester M20 9BX

SUMMARY Using an antiserum raised against tumour angiogenesis factor (TAF) we have developed a radioimmunoassay for retina and tumour angiogenesis factor(s). This antiserum was previously shown to bind to both human and animal tumour extracts and to inhibit the angiogenesis induced by TAF *in vivo*. TAF from rat Walker tumour was used for iodination by the chloramine-T method. An excess of ^{125}I -labelled TAF was incubated with TAF antibody in the absence (maximum binding) and presence (inhibition of maximum binding) of unlabelled tissue extract. A double antibody technique was used to separate free and bound TAF. Unlabelled human Wilms tumour TAF was used as a standard. The extent of inhibition of ^{125}I -TAF-anti-TAF binding provided a measure of TAF in tissue extracts examined. Extracts of normal bovine retina, cornea, lung, aorta, lymph nodes, iris, vitreous humour, and human tumours and normal human pituitary and liver were assayed. Only bovine retina and human tumours were found to contain angiogenesis factor. These findings, together with our earlier results, suggest that angiogenesis factor from both bovine retina and human tumours induce angiogenesis *in vivo* and possess common antigenic determinants. The presence of angiogenesis factor in healthy retina and its relationship to neovascularisation in clinical conditions is discussed.

Over a decade ago Folkman and his associates demonstrated angiogenesis activity in semipurified extracts of solid tumours.¹ A number of other investigators have also found such an activity in non-tumour sources, particularly retina.²⁻⁹ For instance, Federman *et al.* reported the growth of limbal vessels in response to retinal tissue implants in rabbit cornea.⁵ More recently extracts of retinal tissues of many species have been shown to contain angiogenesis factor.³⁻⁹ We have also purified an angiogenesis factor from healthy feline³ and bovine retinas (unpublished data) which appears to be similar to those isolated previously by us from tumours and synovial fluid of patients with joint disease.² However, the interrelationship and differences between various angiogenesis factors remain unknown. In an earlier report we described the development of 2 immunoassays, radioimmunoassay (RIA) and radioimmunometric assay (RIMA), to quantitate angiogenesis factor in tumour extracts.¹⁰ In this communication we have used RIA to quantitate angiogenesis factor in bovine retina and tumour extracts.

Correspondence to Dr S. Kumar.

Materials and methods

RIA buffer. 0.1 M phosphate buffer (pH 7.6) containing 0.15 M NaCl, 0.05% sodium azide and 0.5% bovine serum albumin (Sigma Fraction V Powder).

Tumour angiogenesis factor (TAF). TAF was extracted from rat Walker and human Wilms tumours according to the method described by Phillips and Kumar¹¹ and stored lyophilised at 4°C. The angiogenic substance, although only partially pure, will be referred to as tumour angiogenesis factor (TAF). The TAF was tested by a chorioallantoic membrane (CAM) assay.¹¹ Fig. 1 shows a control CAM and 2 other CAMs treated with bovine retina angiogenesis factor and tumour angiogenesis factor. Both tumours and retina angiogenesis factors induced similar stimulation as shown by the spoke-wheel convergence of blood vessels towards the site of TAF application.

Bovine tissue extracts. For each extraction 20 bovine retinas (approximately 10 g) were collected from the local abattoir and transported to the laboratory in crushed ice. The extraction procedure involved homogenisation of the tissue in RIA buffer

Fig. 1 Chick chorioallantoic membrane: (a) control, (b) showing neovascular response to retinal angiogenesis factor, and (c) tumour angiogenesis factor. In both (b) and (c) newly formed blood vessels converge towards the site of angiogenesis factor application.

(1 g/5 ml) for 3 minutes with a Silverson sealed unit homogeniser. The homogenate was further ultra-sonicated for 1 minute (2 bursts each lasting 20 seconds). Sonication was followed by centrifugation at 2000 g for 15 minutes at 4°C. The supernatant was used for the assay. Normal bovine cornea, lymph node, aorta, and iris were extracted similarly to retinas. The vitreous humour of 3 bovine eyes was pooled and tested by the same assay.

Human tumour and nontumour extracts. Human tumour tissues were surgical biopsy specimens. Normal human pituitary and liver were autopsy material obtained within a few hours of death. All tissues were stored frozen (-20°C) until required.

Extracts of both tumour and nontumour tissue were prepared by the same method as that used for bovine tissue. Protein was estimated by the method of Lowry *et al.*¹²

TAF antiserum. An antiserum was raised in a rabbit against rat Walker TAF as described earlier.¹¹ It was found to bind to a variety of human and animal tumours (detected by gel diffusion and immunofluorescence) and to inhibit the angiogenesis induced by TAF *in vivo*.

Iodination of TAF. Rat Walker TAF was labelled with ¹²⁵I by the method of Hunter and Greenwood.¹³ All the solutions were made in phosphate-buffered saline (PBS) (0.05 M pH 7.6) The radioiodination was

Fig. 2 Binding of ¹²⁵I rat Walker TAF to TAF antiserum. 50 µl of labelled TAF (50 000 cpm) was incubated with different dilutions of TAF antiserum in a total volume of 400 µl. The bound ¹²⁵I-TAF was precipitated with a second antibody (see 'Materials and methods').

carried out at room temperature in a small glass tube placed on a magnetic stirring table in the fume cupboard. Walker TAF (2.5 μg) was mixed with 1 mCi Na^{125}I and 0.1 mg chloramine-T in a total volume of 50 μl . The reaction mixture was agitated continuously by a metal paper clip. The iodination reaction was stopped after 20 seconds by adding successively 100 μl of sodium metabisulphite (34 mg/ml) and KI (10 mg/ml). The free iodine was separated from labelled TAF by gel filtration with a Sephadex G-50 column equilibrated with PBS. Fractions were collected in tubes containing RIA buffer. The radioactivity of each fraction was measured in a gamma counter.

TCA precipitation for the estimation of ^{125}I -labelled protein. In order to estimate the concentration of ^{125}I associated protein 50 μl of the fraction of each peak was mixed with 450 μl of RIA buffer and 500 μl of 10% TCA solution. After rapid mixing, the tubes were incubated for 30 min and centrifuged at 2000 g for 30 min at 4°C . The supernatants were discarded and the radioactivity in the pellet was measured.

Competitive binding of labelled TAF and unlabelled tissue extracts to the TAF antiserum (radioimmunoassay). The binding of ^{125}I Walker TAF to serial dilutions of the TAF antiserum and the competition between the labelled TAF and unlabelled tissue extract for the antibody binding sites was studied by the standard radioimmunoassay (RIA) technique.¹⁴ A double-antibody technique was used to separate bound and free antigen.¹⁵ Unless specified, dilutions were made in the RIA buffer. All assays were

performed in LP3 polystyrene tubes (Luckham Ltd., Sussex, England) and reagents added with Eppendorff pipettes fitted with disposable plastic tips. Duplicate samples were used for every assay. The details of RIA are as published previously.¹⁰ 50 μl of a ^{125}I Walker TAF (containing 30 000–60 000 cpm), 100 μl of TAF antiserum diluted to give a binding with 50% of the iodinated TAF, 100 μl of buffer (to estimate maximum binding), or 100 μl of serial dilutions of Wilms TAF (to obtain a standard curve), or the tissue extracts to be tested and 150 μl of normal rabbit serum (diluted 1:300) were mixed rapidly. After 16–20 h incubation at 4°C , 150 μl of anti-rabbit precipitating serum (diluted 1:10) (RD17, Wellcome Reagents, Beckenham, Kent, England) was added. To estimate the concentration of protein-associated ^{125}I , 150 μl of 40% TCA solution was added to the controls instead of the anti-rabbit precipitating serum. The tubes were incubated overnight at 4°C , followed by centrifugation at 2000 g for 30 min at 4°C . The supernatant was discarded and the radioactivity in the residues was measured in a gamma counter.

Results

Gel filtration of different batches of ^{125}I -labelled Walker TAF produced 3 or 4 peaks. The percentage of ^{125}I protein (TCA precipitable) varied between 94% in the first to about 4% in the fourth batch. Peak 1 was found to bind to TAF antiserum. In a few instances peak 2 also bound to antiserum. In such

Fig. 3 Inhibition of ^{125}I rat Walker TAF-anti-TAF binding by human Wilms TAF. ^{125}I rat Walker (45 000 cpm) was incubated with TAF antiserum diluted 1:1600 in a total volume of 400 μl . In the absence of unlabelled TAF, 25% of the TCA-insoluble ^{125}I was bound to the antiserum. Binding in the presence of different concentrations of Wilms TAF is expressed as a percentage of that maximum binding.

Fig. 4 Inhibition of ^{125}I rat Walker TAF-anti-TAF binding by various tissue extracts. Experimental details were the same as those in Fig. 3.

cases the first 2 peaks were pooled. The specific activity ranged between 10 and 14 $\mu\text{Ci}/\mu\text{g}$ TAF protein. Antiserum titration was carried out by incubating a constant amount of ^{125}I Walker TAF with different dilutions of the antiserum (1:125 to 1:10 000).

Fig. 2 illustrates the antiserum titration curve. At least duplicate samples were used for every assay; the standard deviations were less than 10% of the mean. The inhibition of ^{125}I Walker TAF and anti-TAF

binding by unlabelled Wilms TAF provided a standard curve for the assay.

Fig. 3 shows 2 standard curves obtained by two different batches of Wilms TAF. The displacement induced by the unlabelled tissue extracts provided the inhibition curves which were compared with the standard curve. There was parallelism between the standard curve produced by Wilms TAF and the inhibition curves produced by both bovine retina and

Table 1 Tissue samples were extracted and assayed by RIA for angiogenesis factor, and the results were expressed in terms of wet weight and protein weight as described in the text

Tissue extract	Numbers examined	TAF	
		$\mu\text{g/g}$ wet wt.	$\mu\text{g}/\mu\text{g}$ protein
Bovine			
Retina	3	2640; 1986; 378	0.11; 0.06; 0.0045
Cornea	3	0	0
Lung	3	0	0
Lymph node	3	0	0
Aorta	3	0	0
Iris	2	0	0
Vitreous humour	2	0	0
Human			
(a) Tumours			
Wilms	3	1134; 1260; 718	0.018; 0.02; 0.104
Osteochondroma	1	2025	0.016
Hypernephroma	1	1344	0.028
Lymphoma	1	48	0.001
Glioma	2	869; 665	0.01; 0.0075
Fibrosarcoma	1	370	0.017
(b) Normal tissues			
Pituitary	1	54	0.001
Liver	1	65	0.001

Fig. 5 Bovine and human tissue extracts were assayed for angiogenesis factor by RIA. The levels of angiogenesis factor in different types of tumour and nontumour tissue extracts are compared. The number of different tissue extracts examined is indicated in parentheses.

human tumour extracts (Fig. 4). This indicated an immunological identity between Wilms TAF and the extracts of bovine retina and human tumours.

The quantity of angiogenesis factor in a tissue extract producing an inhibition of the maximum binding was expressed as the quantity of Wilms TAF

Table 2 Amounts of various extracts (μg protein) needed to obtain 25% inhibition of ^{125}I -TAF-anti-TAF binding

Source of extract	Numbers examined	μg Protein
Bovine		
Retina	3	9.5; 19; 370
Cornea	3	>690; >790; >720
Lung	3	>1380; >920; >1170
Aorta	3	>960; >920; >860
Lymph node	3	>1200; >970; >850
Iris	2	>750; >770
Vitreous humour	2	>190; >110
Human		
(a) Tumours		
Wilms's	3	70; 65; 125
Osteochondroma	1	80
Hypernephroma	1	45
Lymphoma	1	>1000
Fibrosarcoma	1	75
Glioma	2	130; 170
(b) Normal tissue		
Pituitary	1	>1050
Liver	1	>1300

producing similar inhibition. The values for angiogenesis factor were expressed as both μg TAF/g wet weight and μg TAF/ μg protein of the tissue (Fig. 5 and Table 1). The level of angiogenesis factor in retinal extracts varied from 378 to 2640 μg TAF/g wet weight. Extracts of other normal bovine tissues did not inhibit the binding to any significant extent, that is, the angiogenesis factor if present in these tissues could not be detected by the present RIA. Considerable inhibition was produced by all human tumour extracts but not by either normal human pituitary or liver. The values for different tumour types varied from 48 to 2025 μg TAF/g wet weight.

The detection limits (defined as the amount of protein producing 25% inhibition of the maximum binding) of the assay are shown in Table 2. 9.5–370 μg protein of the retinal extracts and 45–170 μg protein of the human tumour extracts were required to produce 25% inhibition.

Discussion

We have developed a radioimmunoassay (RIA) for an angiogenesis factor from bovine retina using an antiserum raised against rat Walker TAF. This antiserum has been previously reported to bind to human and animal tumours (detected by gel diffusion and immunofluorescence) and to inhibit TAF induced angiogenesis in vivo.¹¹ Our data show that TAF and the extracts of bovine retina and human tumours possess common antigenic determinant(s), which are either absent or present only in much smaller concentrations in normal human pituitary and liver and bovine aorta, lung, cornea, iris, lymph node, and vitreous humour. The inhibition curves produced by retinal and tumour extracts were parallel to those produced by Wilms TAF. The amounts of angiogenesis factor, estimated as equivalent of Wilms TAF, were equal or slightly more in bovine retinal extracts compared with tumour extracts. These findings confirm and significantly extend our previous work.³ Partially and highly purified extracts from tumours and healthy feline³ and bovine retinas (unpublished data) were found to give an angiogenic response on the chick chorioallantoic membrane. These extracts also bound to TAF antiserum linked to a sepharose affinity column. Furthermore, D'Amore *et al.* have also isolated an angiogenesis factor from normal bovine retina.⁹ The structural identity of tumour or retinal angiogenesis factor(s) is not known.

For a solid tumour to grow beyond a size of a few cubic mm it needs the formation of new blood vessels, and thus the role of angiogenesis factor in tumours is obvious. Why a large amount of angiogenesis factor is present in normal retina and why its presence does not induce angiogenesis requires an explanation

Henkind has suggested that, for retinal neovascularisation to occur, both an abnormal retinal vascular bed and a vasculogenic factor produced by a hypoxic retina are required.¹⁶ If vascular perfusion is relatively good the retinal vessels will not proliferate, even though a vasculogenic substance is present.

Another explanation for the absence of neovascularisation of healthy retina may be that vitreous humour has been shown to contain substances that inhibit the growth of new blood vessels by tumour angiogenesis factor.¹⁷⁻¹⁹ Such inhibitors may be involved in controlling the activity of any retinal angiogenesis factor. It is also possible that healthy retina itself may contain inhibitors in addition to activators (TAF) to maintain an equilibrium. The marked variation in the quantity of angiogenesis factor among 3 retinal extracts (see Table 1) can be explained by the above conditions.

Diabetic retinopathy, retrolental fibroplasia, and sickle cell retinopathy are common examples of clinical conditions where retinal neovascularisation occurs. The likeliest cause of these conditions seems to be venous occlusion or ischaemia resulting in hypoxic retinal tissue. As mentioned earlier, Henkind has suggested that such hypoxic tissue can liberate a vasculogenic stimulator. The proposed vasculogenic stimulator may be the same as angiogenesis factor.^{16 20 21}

In summary, using a radioimmunoassay we have demonstrated for the first time a bovine retina-derived substance immunologically identical to tumour angiogenesis factor. The role of angiogenesis factor in diseases where retinal neovascularisation occurs needs investigation.

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