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Original Paper

Tumour Suppressor Genes and Angiogenesis: the Role of *TP53* in Fibroblasts

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INTRODUCTION

AS NORMAL cells develop the ability to produce solid tumours, they must acquire at least three key characteristics that differentiate them from their normal progenitors. They must become able to divide easily under conditions where normal cells are either quiescent or dividing in an orderly manner. They must acquire the ability to attract new blood vessels to support their progressive expansion, and they must avoid clearance by the immune system as they undergo these changes. These alterations in phenotype occur as a result of genetic lesions that activate oncogenes and inactivate tumour suppressor genes. There are now more than 50 oncogenes identified. The list of human tumour suppressor genes stands at 16 and is rapidly growing. Thus, there exists an immense number of possible combinations of activated oncogenes and inactive tumour suppressor genes that could, in theory, lead to malignancy.

Yet in any single type of human tumour, specific lesions occur in only a restricted subset of oncogenes and tumour suppressor genes. Most colon carcinomas have lost tumour suppressor genes *APC, TP53* and *DCC* and activated oncogene *K-RAS* [1]. The majority of pancreatic cancers activate *RAS* and lose the tumour suppressor *DPC* [2]. The specific combination of activated oncogenes and inactivated tumour suppressor genes necessary and sufficient to produce a tumour in a given tissue is determined by sequential rounds of clonal selection among the many spontaneously arising mutant clones within the developing tumour [3]. The surprising degree of predictability in a given tissue seems to reflect tissue-specific constraints arising from (i) a limited subset of genetic lesions available for selection to act on, which in turn depends on mutagenic influences specific to that tissue and the sensitivity of the various oncogenes and tumour suppressor genes to such mutagens, and (ii) the strength of the advantage a lesion in a given oncogene or tumour suppressor gene confers on a cell in that particular tissue. The distinct selective pressures that direct such tumour evolution along different pathways in different tissues come from a variety of sources and are analogous to the constraints imposed by the 'soil' in which a new metastasis finds itself (see Ellis and Fidler, pages 2451–2460). In both cases, the ability to induce angiogenesis plays an essential role.

All available data on tumour growth *in vivo* (see [4]) indicate that an incipient tumour can expand to a size of clinical importance only if it is able to induce angiogenesis. Thus, in a given tissue, one would not expect a successful tumour to arise unless its cells contained a combination of activated oncogenes and inactivated tumour suppressor genes that was sufficient to enable it to be potently angiogenic in that environment. In mouse models and in naturally occurring human tumours (see [5]), an angiogenic phenotype develops during tumour progression, while oncogenes are activated and tumour suppressor genes lost. Sometimes vessel density increases gradually as progression occurs, as in cervical squamous cell carcinoma. At other times, it appears to increase suddenly as in breast carcinomas. The selective power of angiogenic activity *in vivo* is reflected in hot spots of increased vessel density that occur frequently in human tumours and are harbingers of poor prognoses [6].

In vitro studies convincingly demonstrate that specific oncogenes and tumour suppressor genes can dramatically influence angiogenesis [7, 8]. Cultured normal cells that have no activated oncogenes and retain all their tumour suppressor genes are not angiogenic. In most instances, they are actually anti-angiogenic. Normal keratinocytes, fibroblasts, retinal cells and glial cells all secrete mixtures of molecules that, when tested in angiogenesis assays, are inhibitory [7]. The introduction of activated oncogenes and the inactivation of tumour suppressor genes can change this phenotype to that of cultured tumour cells whose secretions are potently angiogenic [9].

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Oncogenes influence angiogenesis by encoding secreted proteins that are themselves angiogenic factors or by stimulating cells in which they are expressed to secrete increased levels of angiogenic factors as well as a variety of enzymes that enhance angiogenesis [7]. For example, activated *RAS* oncogenes expressed in fibroblasts [9] or epithelial cells [10] *in vitro* stimulate the secretion of the angiogenic molecule, vascular endothelial cell growth factor (VEGF) and enhance neovascularisation of similar cell types *in vivo* [11, 12].

Although it is clear that oncogene activation has the ability to enhance angiogenesis, it is tumour suppressor gene loss that may have a more profound effect on the angiogenic phenotype of the developing tumour cell. Human tumour suppressor genes that have been shown to influence angiogenesis are listed in Table 1. In most cases, when a wild type tumour suppressor gene is expressed in a cell sensitive to its angiogenic effects, it supports the production of inhibitory activity that is sufficient to block angiogenesis induced by a cognate tumour [13–17]. In several cases, inhibitory activity depends on a single secreted protein. In such cases, loss of the activity of the tumour suppressor gene results in a fall in the secretion of inhibitory protein [13, 14], and return of a wild type tumour suppressor gene to a tumour cell of a sensitive tissue often restores secretion of the inhibitor to a level sufficient to produce an anti-angiogenic phenotype [13–17].

The most widely investigated tumour suppressor gene is *TP53*. When expressed exogenously in human fibroblasts, wild type p53 influences angiogenesis in two ways: it supports the secretion of inhibitory thrombospondin-1 (TSP-1) [13–15] and depresses secretion of the inducer, VEGF [9, 19]. Thus, when *TP53* is inactivated in these cells, they become more angiogenic due both to a decrease in the secretion of inhibitory TSP, which falls by over 20-fold, and to an increase of approximately 4-fold in the secretion of angiogenic VEGF. Of these two effects, the fall in the inhibitor is the more important, for fibroblasts retaining wild type p53 secrete inhibitory activity that is so potent it can be diluted 3-fold and still block angiogenesis induced by tumour cells [9]. The von Hippel Lindau (VHL) tumour suppressor gene has also been shown to depress the induction of VEGF [18, 20]. It is not known if VHL also supports the secretion of an inhibitor.

Whether or not wild type *TP53* influences angiogenesis depends on the cell in which it is expressed. Although in fibroblasts and human breast carcinoma cells it supports the production of inhibitory TSP [13, 15], when returned to cells from a human glioblastoma, it stimulates the production of an unidentified inhibitor that is definitely not thrombospondin [16]. Instead, a tumour suppressor gene located on chromosome 10q is able to stimulate the secretion of TSP in glioblastoma [17]. As the control of TSP by tumour suppressor genes occurs largely at the level of transcription, it is perhaps not surprising that genes able to control its production in one cellular environment fail in another. There is ample evidence that the ability of wild type *TP53* to regulate a variety of genes and their associated functions varies from one cell type to another [21].

The *TP53* tumour suppressor gene has a number of activities in addition to its ability to block angiogenesis, all of which can suppress tumour formation [8]. It can retard the accumulation of DNA lesions, thus decreasing the probability of the activation of oncogenes and loss of tumour suppressor genes. It can directly restrict the growth of incipient tumour cells in a variety of ways of which its ability to mediate apoptosis may be the most relevant. In most cases, these functions of *TP53* require that the low level of active protein found in normal cells be increased by heat, hypoxia, DNA damage or other stresses. These observations, coupled with the fact that *TP53* null animals, although their life span is cut short by a variety of cancers, are otherwise healthy and fertile, has suggested that *TP53* plays a protective role in tumour suppression, springing into action only when provoked to do so by some cellular insult [22].

To determine if the inhibition of angiogenesis by *TP53* via TSP can occur in the absence of such overt insults and if it is strictly dependent on the presence of wild type p53, we compared TSP levels and angiogenic activity in fibroblasts cultured from a variety of mice, including those that were wild type for *TP53* and for TSP-1 and those that were null for genes encoding each of these proteins and hybrids between the two null mice. The results are presented below.

MATERIALS AND METHODS

Cell culture and media

Bovine capillary endothelial cells BP10T8 were a kind gift of Dr J. Folkman (Children's Hospital, Harvard Medical School, Massachusetts, U.S.A.), and were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% donor calf serum and 10 μg/ml endothelial cell mitogen (Biomedical Technologies, Stoughton, Massachusetts, U.S.A.). Murine dermal fibroblasts were prepared as described by Yuspa and Harris [23] from the skin of 1–10 day old pups of wild type mice, *TP53* null

Table 1. Human tumour suppressor genes shown to influence angiogenesis

Tumour suppressor	Cell type	Effect on angiogenesis	Angiogenic mediator	Ref.
TP53	Glioblastoma	inhibitory	? [not thrombospondin]	[16]
	Fibroblasts	inhibitory	Thrombospondin	[13, 14]
		↓induction	VEGF	[9]
	Breast carcinoma	inhibitory	Thrombospondin	[15]
RB	Retinoblastoma	inhibitory	50 kDa protein	*
	Osteosarcoma	inhibitory	?	*
	Prostate	none		*
VHL	Renal cell carcinoma	↓induction	VEGF	[18]
Chromosome 10q	Glioblastoma	inhibitory	Thrombospondin	[17]
Chromosome 17	Neuroblastoma	inhibitory	?	†

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mice [24], *TSP-1* null mice or hybrids between these two null animals that were missing both the *TP53* and the *TSP-1* genes. *TSP-1* null mice were generated by homologous recombination in ES cells (J. Lawler and R.O. Hynes, Brigham and Women's Hospital, Massachusetts, U.S.A.). All mutations were studied on a mixed C5-7BL/6 and 129Sv background. Murine fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum, 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid and ascorbic acid (50 μg/ml) and used prior to passage 3.

Conditioned media collection

Cells were grown to 90% confluence, growth media were aspirated, cells were washed with DMEM, incubated 4–7 h in DMEM, then fresh serum-free DMEM was added. Media were collected 48–65 h later and, after removal of debris, concentrated 5–20-fold using Centricon concentrators with a 3 kDa molecular weight cut-off (Amicon, Beverly, Massachusetts, U.S.A.). Media were used at 20 μg/ml in migration assays and 200 μg/ml in corneal assays.

Antibodies

A monoclonal antibody raised gainst TSP-1, A4.1, known to neutralise its anti-angiogenic activity [25, 26], was used at 30 μg/ml in the migration assays and at 300 μg/ml in the cornea assay. A pan-specific anti-TGF-β polyclonal neutralising antibody (R&D Systems, Minneapolis, U.S.A.) was used at 60 μg/ml in the migration assays.

Vessel counts

The entire eye was fixed in 10% buffered formalin, and embedded in paraffin for cross-sectional analysis by light microscopy. The block was serially sectioned until the appropriate sagittal plane was identified that included cornea, anterior chamber, lens, posterior chamber and retina. All capillary-sized vessels with recognisable endothelial cells were counted in the anterior and posterior eye chambers.

In vitro *angiogenesis assay*

The ability of media conditioned by murine fibroblasts to stimulate or inhibit the migration of bovine capillary endothelial cells was assayed as previously described [27]. DME with 0.1% bovine serum albumin (BSA) was used as a negative control and basic fibroblast growth factor (bFGF) (10 ng/ml) was used as a positive control. Each sample was tested in quadruplicate in a single experiment and experiments were repeated at least twice. Results are expressed as the number of endothelial cells migrating to the side of the sample per 10 high power fields (h.p.f.). When multiple experiments were combined, background migration towards BSA was subtracted and data normalised to the percentage of migration towards bFGF. Standard errors are indicated on the figures

In vitro *angiogenesis assay*

The ability of experimental samples to induce or inhibit neovascularisation in the normally avascular cornea of the rat was assessed as previously described [28]. Briefly, a pellet of less than 5 μl was formulated containing test ingredients in hydroxyethylmethacrylate (Hydron^{R}, Interferon Services, New Brunswick, New Jersey, U.S.A.) and implanted 1–1.5 mm from the limbus of the cornea of an anaesthetised rat. Neovascularisation was assessed 7 days after implantation. Animals were anaesthetised, perfused with colloidal carbon to label vessels, and corneas were excised, flattened and photographed for a permanent record.

Immunoblot analysis

TSP-1 levels in media conditioned by dermal fibroblasts were analysed by subjecting samples containing 15 μg of protein that had been reduced and alkylated [29] to electrophoresis on 4–12% gradient polyacrylamide gels (Novex, San Diego, California, U.S.A.), and blotting the proteins on to Hybond N Nylon membranes (Amersham, Arlington Heights, Illinois, U.S.A.). Blots were incubated with anti-TSP monoclonal antibody, A4.1 [25], which recognises mouse TSP-1 but not mouse TSP-2, and neutralises the anti-angiogenic activity of human and hamster TSP [25–27]. Bound antibody was detected using horseradish peroxidase conjugated goat anti-mouse IgM and visualised using the ECL detection kit from Amersham. Recombinant murine TSP-1 and -2 proteins, produced in a baculovirus system and kindly supplied by Paul Tooney and Deane Mosher, University of Wisconsin, Wisconsin, U.S.A. [30], were used as controls.

RESULTS

The effect of loss of wild type TP53 *on TSP-1 and angiogenesis*

Published data from this laboratory suggest that, in fibroblasts, the tumour suppressor gene *TP53* stimulates the secretion of TSP-1 at levels sufficient to cause the cells to be anti-angiogenic [13, 14]. Experiments supporting this contention used constructs that overexpressed wild type *TP53* in immortal fibroblasts in which the *TP53* gene was inactivated by mutation. If wild type p53 protein is truly essential for TSP-1 production, then removing only this protein from normal, mortal fibroblasts should also be sufficient to decrease the secretion of TSP-1 and switch those fibroblasts (which normally secrete low levels of inducers of angiogenesis [9, 14]) from an anti-angiogenic to an angiogenic phenotype.

To test this, dermal fibroblasts were cultured from mice null for *TP53* and compared to those cultured under similar conditions from wild type mice and from mice null for *TSP-1* itself. To determine the angiogenic phenotype of these cells, secretions were collected as serum-free conditioned media and tested in angiogenesis assays. When the *in vitro* migration assay was used (Figure 1), media collected from cells derived from *TP53* null animals were indistinguishable from those collected from cells derived from *TSP-1* null mice. Unlike media from cells derived from wild type animals, they both induced the migration of capillary endothelial cells, failed to inhibit migration induced by basic fibroblast growth factor (bFGF) and their activities did not change significantly in the presence of antibodies known to neutralise the anti-angiogenic activity of TSP-1 [25]. Cells cultured from double null animals lacking both *TP53* and TSP-1 were similar. Cells cultured from multiple animals of each genotype all behaved similarly. In addition, embryonic fibroblasts derived from a second *TP53* null stock [31], kindly supplied by A. Sands and A. Bradley, also failed to secrete TSP-1 and to display an anti-angiogenic phenotype.

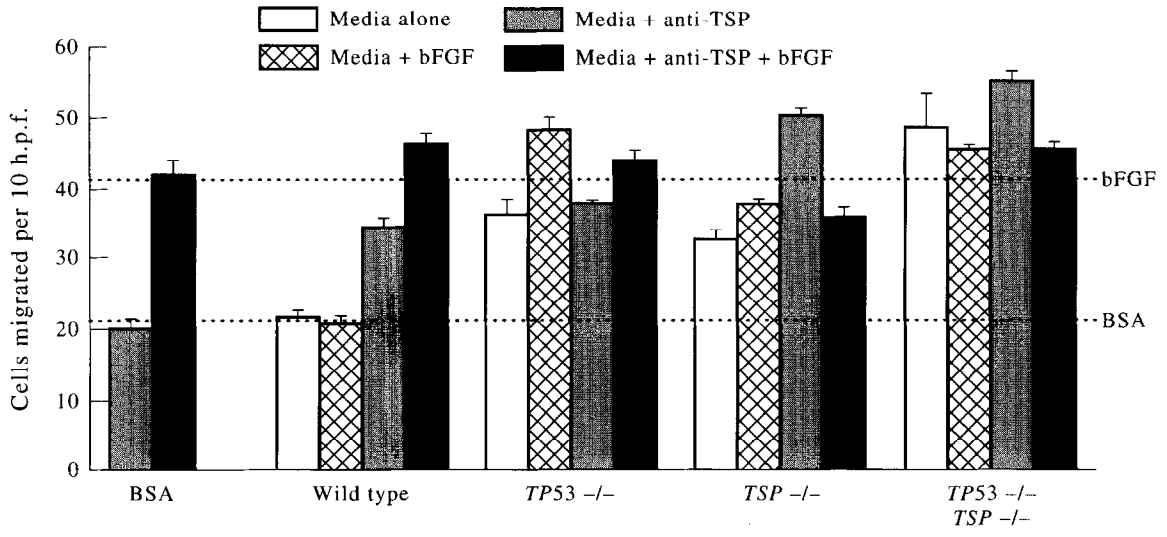

Figure 1. Thrombospondin-dependent inhibitory activity is no longer secreted by fibroblasts null for either *TP53* or *TSP-1*. Media conditioned by fibroblast cultures derived from mice of the indicated genotypes were tested for their ability to induce migration of capillary endothelial cells in the presence of basic fibroblast growth factor (bFGF), neutralising anti-TSP antibodies (anti-TSP) or both. Broken line labelled BSA indicates background migration towards media containing only BSA; that labelled bFGF indicates migration towards media containing BSA and bFGF.

Although TSP-1 can activate TGF-β [32], which is secreted in an inactive form by many cells, such activity was not responsible for the change in angiogenic phenotype seen in null fibroblasts. The angiogenic activity of their media were unaffected by antibodies that neutralised the angiogenic effects of TGF-β (Figure 2).

The angiogenic activity observed *in vitro* using cultured capillary endothelial cells was reproduced *in vivo* in the rat cornea where conditioned media from both *TP53* null and *TSP-1* null fibroblasts induced neovascularisation while that from wild type cells did not (Table 2). The media from these wild type mouse fibroblasts was similar to that collected from normal human fibroblasts [13, 14] in that its inability to stimulate angiogenesis was due to the presence of inhibitory TSP-1 (Table 3).

The similarity in angiogenic phenotype between the *TSP-1* null and *TP53* null animals seems likely to be due to the decreased secretion of TSP-1, since Western blots confirmed that only wild type cells secreted high levels of this protein (Figure 3). Similar results were obtained with cells cultured from additional animals. A second monoclonal antibody raised to TSP-1, A6.1 [25], also showed a similar pattern for TSP-1 in *TP53* and *TSP-1* null aminals.

The influence of wild type TP53 *on the angiogenic activity of serum*

Platelets are a major source of TSP-1 *in vivo* [33] and their degranulation accounts for the high levels of this protein in sera. To assess the contribution of TSP-1 to the angiogenic activity of serum and to determine if it was influ-

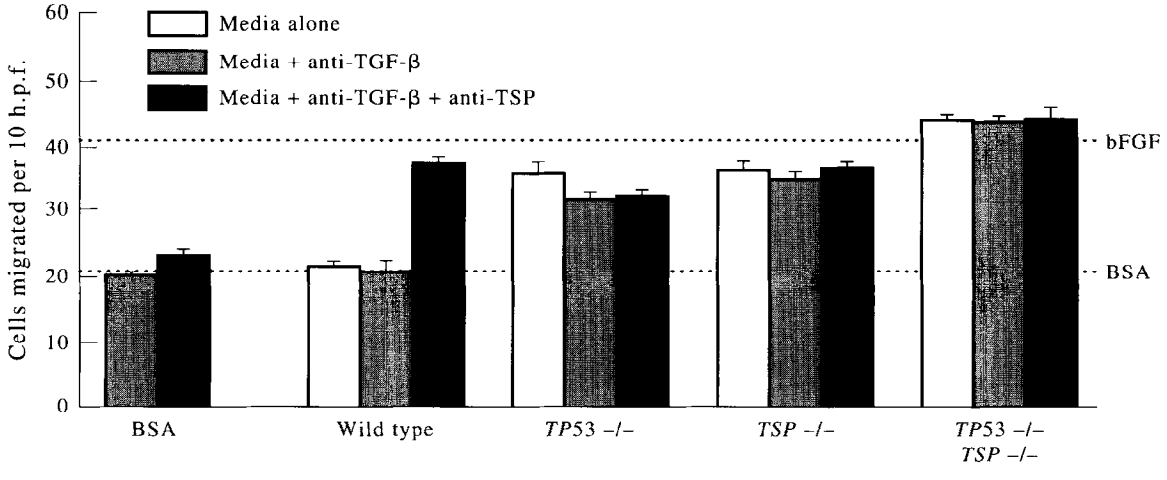

Figure 2. Changes in secreted angiogenic activity of fibroblasts are independent of TGF-β. Media conditioned by fibroblasts of the indicated genotypes were tested for the ability to induce capillary endothelial cell migration in the presence of neutralising antibodies directed against TGF-βs or against the TGF-βs and TSP. Broken lines as in Figure 1 caption. In control experiments, anti-TGF-β antibodies were effective at blocking the ability of 0.5 ng/ml of TGF-β to inhibit the migration of endothelial cells towards stimulatory VEGF.

Table 2. Effect of murine fibroblast conditioned media on corneal neovascularisation

Fibroblast genotype *TP53*	Fibroblast genotype *TSP-1*	*TSP-1* secreted*	Positive corneas/ number implanted
+	+	+ + + +	0/3
–	+	+/–	2/3
+	–	–	3/3
–	–	–	3/3

* See Figure 3.

Secretions were collected from dermal fibroblasts cultured from mice that were wild type (+), null (–) for *TP53* and/or for *TSP-1*, concentrated and incorporated into pellets that were implanted in rat corneas. Vigorous growth of vessels into the normally avascular cornea at 7 days was scored as a positive response.

enced by wild type p53, sera from wild type, *TP53* null and *TSP-1* null animals was collected and tested in dose–response curves using the capillary endothelial cell migration assay (Figure 4). Distinct differences were seen. Serum from wild type mice failed to show any angiogenic activity until concentrations were increased to >100 μg/ml of the total protein (Figure 4a). When tested with stimulatory concentrations of bFGF (Figure 4a), clear inhibitory activity was seen, that increased dose-dependently, persisted as the protein concentration increased over a 500-fold range, and then broke down at serum concentrations at or above 300 μg/ml.

As was the case with fibroblast media, the sera derived from mice null for *TP53* or for *TSP-1* were similar to one another in angiogenic activity and were significantly different from serum from wild type mice. Sera from both null animals were able to stimulate angiogenesis over a wide range of concentrations where sera from wild type mice were ineffective (Figure 4b,c). They also failed to inhibit angiogenesis stimulated by bFGF at any concentration tested (Figure 4b,c).

The influence of loss of wild type TP53 *on vessel density in the eye*

Eyes of animals of each genotype were fixed and capillary-sized vessels present in the anterior and posterior chambers counted, using a single central sagittal section for each eye to avoid counting the same vessel more than once. Both null animals had significantly more vessels than their wild type counterparts (Table 4).

Table 3. Dependence of the inhibitory activity of wild type fibroblast conditioned media (CM) on thrombospondin

Additions to wild type fibroblast CM: bFGF	Anti-TSP-1	Positive corneas/ number implanted
–	–	0/3
+	–	0/3
–	+	3/3
+	+	3/3

Media conditioned by dermal fibroblasts cultured from wild type mice was tested for angiogenic activity in the rat cornea (see Table 2 footnotes). Where indicated, stimulatory bFGF or neutralising anti-TSP-1 antibodies were added to the pellets.

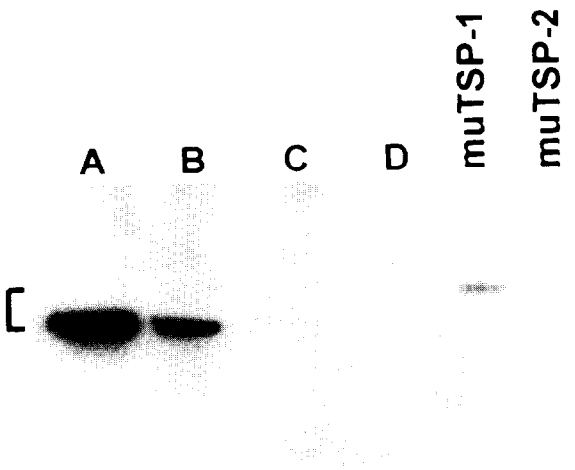

Figure 3. Fibroblasts cultured from animals null for either *TP53* or *TSP-1* fail to secrete high levels of TSP-1 protein. Western blot analysis of TSP-1 present in media conditioned by fibroblasts cultured from mice that were wild type (A), null for *TP53* (B), null for *TSP-1* (C) or null for both *TP53* and *TSP-1* (D). Recombinant proteins mouse thrombospondin-1 (muTSP-1) and mouse thrombospondin-2 (muTSP-2) served as controls. The square bracket on the left indicates TSP-1.

DISCUSSION

Data presented above demonstrate that wild type *TP53* expressed at natural levels in mouse fibroblasts supports the secretion of TSP-1 which maintains their anti-angiogenic phenotype. Wild type p53 also seems likely to do this in human fibroblasts since, at early passages, they are also anti-angiogenic due to TSP-1 secretion [13]. Normal ambient levels of p53 seem to be sufficient for this effect. Mouse fibroblasts were anti-angiogenic in the absence of any treatments to increase the activity of the p53 protein. Although p53 activity was not measured directly, media were collected at early passage, under normoxic conditions at 37°C in buffered media in the absence of any treatments that might induce DNA damage. In human fibroblasts, the maintenance of TSP-1 secretion may also require only modest levels of wild type p53, for these cells remain anti-angiogenic even when expressing one wild type *TP53* allele and one mutant allele that encodes a p53 protein capable of dominant negative effects [13]. A close analysis of such fibroblasts [9] indicates that cells heterozygous for wild type and mutant *TP53* make the same amounts of inducers of angiogenesis and of TSP-1 as do cells homozygous for wild type *TP53*.

It is clear from the different angiogenic profiles of the mouse sera and the different vessel counts in the eyes that loss of *TP53* and loss of *TSP-1* can have similar effects in the whole animal. The simplest explanation for the loss of inhibitory activity in the mouse sera is that wild type *TP53* is required to support the synthesis of TSP-1, the major protein of the platelet alpha granule. Without p53, the platelets, and hence the serum into which they degranulate, are deficient in TSP-1 and thus in anti-angiogenic activity. If verified, this would suggest that TSP-1 is the major inhibitor of angiogenesis in the serum, where it may play a role in delaying the angiogenesis that supports wound healing. The increased vessels present in the eyes of *TSP-1* and *TP53* null animals suggests that TSP-1 may exert a controlling in-

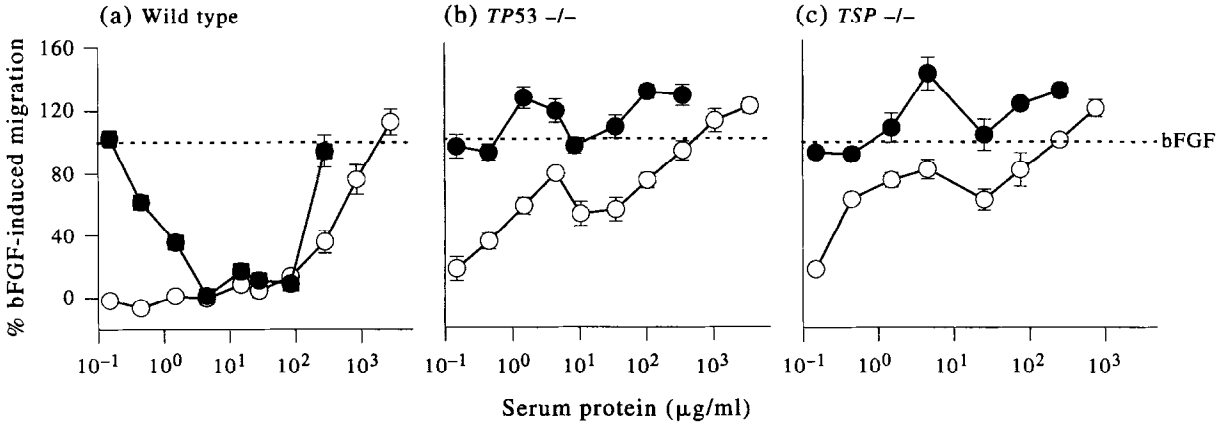

Figure 4. Angio-inhibitory activity of serum from wild type mice is absent in serum from animals null for *TP53* or for *TSP-1*. Serum from mice of the indicated genotypes were collected, diluted and assayed either alone (○) or in the presence of bFGF (●) for ability to induce the migration of capillary endothelial cells.

fluence on ocular vascularisation. TSP-1 is synthesised by several cell types that are in contact with the eye chambers [34, 35] and could be under *TP53* control in these tissues.

Simple lack of wild type p53 was sufficient to switch mouse fibroblasts from an anti-angiogenic to an angiogenic phenotype. Although this switch was verified by testing conditioned media in the *in vivo* cornea assay, it is not yet possible to say whether or not in a developing tumour *in vivo* loss of p53 would be sufficient to cause a sudden increase in neovascularisation. If the surrounding tissue is high in inhibitors, it may require both loss of TSP-1 and an increase in inducing molecules before an impact is seen.

Most human fibrosarcomas have lost the activity of wild type p53, as might be expected from the angiogenesis data presented above. However, some retain the wild type allele. When one of these human tumour lines was examined recently [9], it was found to be angiogenic despite making high levels of inhibitory TSP-1. The TSP-1 was overcome by the secretion of extremely high levels of angiogenic factors. bFGF and VEGF were produced by these cells at 10 times the level seen in tumorigenic fibroblasts that lost wild type p53. Thus, even in a single tumour type, the fibrosarcoma, there is more than one way to achieve an angiogenic phenotype sufficient to support tumour growth *in vivo*.

Yet it seems likely that what suffices for a fibrosarcoma might not be sufficient for a tumour of another tissue type. Different tumour types tend to rely on different angiogenic factors, for example, IL-8 for squamous cell carcinoma, VEGF for glioblastoma, and different tissues have different inhibitors that must be overcome, for example TIMP (tissue inhibitor of metalloproteinase) in cartilage, a different protein in the cornea. In addition, genetic changes, such as the loss of *TP53*, can influence angiogenesis in different ways in different tissues. Only further experiments will tell how strong an influence such tissue-specific constraints on angiogenesis have in selecting the genetic changes that underlie tumour-progression.

Table 4. Vessel density is increased in the anterior and posterior chambers of the eyes of TSP-1 *and* TP53 *null animals*

Genotype	*n*	Total vessels/eye
Wild type	3	15 ± 3.5
TP53 null	8	45 ± 3.0
TSP-1 null	6	74 ± 7.7*

Total vessels present in anterior and posterior eye chambers were counted in a single sagittal section through the centre of the eye. *Indicates values that differ significantly from wild type by Wilcoxon rank sum test ($P < 0.05$).

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