

## Tumour Interstitial Fluid Pressure May Regulate Angiogenic Factors in Osteosarcoma

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### Abstract

**Purpose:** We have previously shown that osteosarcomas have states of increased interstitial fluid pressure (IFP) which correlate with increased proliferation and chemosensitivity. In this study, we hypothesized that constitutively raised IFP in osteosarcomas regulates angiogenesis. **Materials and Methods:** Sixteen patients with the clinical diagnosis of osteosarcomas underwent blood flow and IFP readings by the wick-in-needle method at the time and location of open biopsy. Vascularity was determined by capillary density in the biopsy specimens. We performed digital image analysis of immunohistochemical staining for CD31, VEGF-A, VEGF-C and TPA on paraffin-embedded tissue blocks of the biopsy samples. Clinical results were validated in a pressurised cell culture system. **Results:** IFPs in the tumours (mean 33.5 ± SD 17.2 mmHg) were significantly higher ( $P = 0.00001$ ) than that in normal tissue (2.9 ± 5.7 mmHg). Pressure readings were significantly higher in low vascularity tumours compared to high vascularity tumours ( $P < 0.001$ ). In the osteosarcoma cell lines, growth in a pressurised environment was associated with VEGF-A downregulation, VEGF-C upregulation and TPA upregulation. The reverse was seen in the OB cell lines. Growth in the HUVEC cell line was not significantly inhibited in a pressurised environment. Immunohistochemical assessment for VEGF-A ( $P = 0.01$ ), VEGF-C ( $P = 0.008$ ) and TPA ( $P = 0.0001$ ) translation were consistent with the findings on PCR. **Conclusion:** Our data suggest that some molecules in angiogenesis are regulated by changes in IFP.

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**Key words:** Angiogenesis, Lymphangiogenesis

### Introduction

Solid tumours have states of raised interstitial fluid pressure (IFP) that cause significant changes to their physiology.<sup>1-3</sup> We have previously shown that human osteosarcomas respond to this raised IFP by increasing their proliferative state. This proliferative state results in increased sensitivity to chemotherapy – a finding noted both in the in vitro setting in osteosarcoma cells grown in a physiologically replicated high pressure environment and the in vivo setting in a series of patients with osteosarcoma who had chemotherapy.<sup>1,4</sup>

Tumour perfusion is reduced by reduced vessel density and reduced blood flow within individual vessels. It has been postulated that blood flow within tumours is reduced by increased IFP in a Starling resistor model although establishment of a good model to prove this has been challenging.<sup>2</sup> Alternatively, we hypothesised that reduced perfusion within high-pressure tumours was due to reduced vascularity as a result of raised IFP. The primary aim of the present study was to investigate this hypothesis through an analysis of human subjects with osteosarcomas. We verified these in vivo findings by investigating the growth of cells

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in a previously described cell culture system that permits cell growth in a pressurised system.<sup>1,4</sup> It has been shown that vascular endothelial growth factor A (VEGF-A) is a pro-angiogenic factor.<sup>5</sup> Tissue plasminogen activator (TPA) indirectly inhibits angiogenesis by increasing angiostatin levels.<sup>6</sup> We analysed VEGF-A and TPA expression in osteosarcoma for their response to pressure.

The permeative microvasculature of these solid tumours leading to leakage of protein into the interstitium raises oncotic pressure and IFP.<sup>7</sup> Other potential causes of increased IFP include interstitial fibrosis and the contraction of fibroblasts within the interstitium of the tumour. This is complicated further by abortive lymphatic development in the depth of these tumours and hence there is no viable outflow for the excessive fluid accumulation.<sup>8</sup> The result is impairment of macromolecule drug transport within this relatively stagnant interstitial space.<sup>9</sup> Hence, as a secondary aim of this study, we investigated the effect that raised IFP has on lymphatic proliferation. Vascular endothelial growth factor C (VEGF-C) has been correlated with lymphangiogenesis.<sup>10</sup> We analysed the effect of IFP on this transcription factor.

## Materials and Methods

### *Clinical Study*

Between January 1997 and June 2000, 16 patients with the clinical diagnosis of high-grade osteosarcoma underwent open biopsy with IFP and blood flow measurements at the biopsy site – the specific techniques of which have been described.<sup>2</sup> Briefly, IFP was measured using a wick-in-needle probe and a Camino 420 digital pressure monitor to a sensitivity of 0.1 mmHg (Camino Laboratories, San Diego, CA). Blood flow within tumours was measured by a Vasamedics BPM 403 Laserflo laser Doppler flow perfusion monitor attached to a 0.8-mm diameter probe (TSI, Inc, Missoula, MT). Readings were taken about 1 cm deep to the tumour surface and from the neighbouring soft tissue. Following diagnosis, the patients were treated with an 8-week course of preoperative chemotherapy using standard regimes. They were then restaged and a definitive resection and reconstruction for their tumours was done. The institutional review board granted approval for the use of clinical data.

Vascularity as seen in the biopsy samples was assessed in the first instance by an observational 4-grade score (Fig. 1). The senior pathologist and lead author would agree upon 3 areas that were representative of the vascularity of the slide as a whole and grade these areas collectively. Both parties were blinded to the identity of the slide. It was not possible to define the gestalt of the score numerically. In general, however, grade 1 tumours were nearly devoid of vascularity, grade 2 tumours had more than 90% of the

high power field occupied by tumour, grade 3 tumours had greater than 50% of the field occupied by tumour and grade 4 tumours had less than 50% occupied by tumour – the remainder being occupied by anatomically definable vessels. IFP and blood flow readings obtained at the time of biopsy were correlated with tumour vascularity and percentage immunohistochemistry staining as described subsequently.

### *Cell Culture System*

All experiments were performed using a cell culture system, the Opticell® (Biocrystal, Westerville, OH) which was modified to permit cell culture in a pressurised environment.<sup>1,4</sup> Cells are grown as monolayers within the system and pressurised using a fluid column which was adjusted to provide 0, 20 or 50 mmHg of static pressure.

We used 2 human osteosarcoma cell lines, HOS and U2OS (ATCC, Manassas, VA) and compared this with a non-neoplastic osteoblast cell line HOb (Cell applications, San Diego, CA). Cells were grown at 3 pressures: 0 mmHg (comparable to conventional culture systems), 20 mmHg (an intermediate osteosarcoma pressure observed in clinical studies) and 50 mmHg (the higher pressures observed in these studies<sup>1,2,11</sup>). For each cell line, the number of cells in each chamber was the same at all 3 pressures. Cells were grown under pressure for 72 hours.

To investigate the influence of applied pressure on endothelial cell proliferation, we grew human umbilical vein endothelial cells (HUVEC, ATCC, Manassas, VA) under 0 and 20 mmHg of pressure. Each Opticell® was seeded with  $1.5 \times 10^5$  cells and pressurised for 24, 48 and 72 hours. HUVEC growth under pressure was assessed by counting cells over five 100x high power fields at the beginning and end of the experiment. Growth was expressed as the ratio of the number of cells per high power field at the end of the experiment to that at the beginning of the experiment as previously described.<sup>1</sup> At the end of each time point, cells were removed from the adherent surface using 3 mL of 0.25% trypsin-EDTA and counted with a hemocytometer (Bright-Line®, Horsham, PA) using Trypan blue® stain (Fisher Scientific, Chicago, IL) non-uptake as a marker of cell viability. These data were used as corroborative data for the high power field data. The experiment was done in triplicate.

### *Cell Cycle Analysis*

HUVEC cell lines were pressurised for 24, 48 and 72 hours. They were then prepared for cell cycle analysis. They were fixed in 66% methanol and 33% PBS, then treated with 100 U/mL ribonuclease and DNA fluorescent labelled with 0.05 mg/mL propidium iodide. Cell cycle parameters were measured by flow cytometry using the FACSCalibur System (BD Biosciences, Franklin Lakes, NJ). Data were

analysed using FlowJo 4.3.1 (Tree Star, Ashland, OR). Cell cycle analysis was performed by investigating the FL2-A profile of the cells identified to be in cycle (Fig. 2).

#### *Qualitative Polymerase Chain Reaction*

We used qualitative polymerase chain reactions (PCR) to screen for the presence of VEGF-A, VEGF-C and TPA in the HOS, U2OS and HOb cell lines. Cells were pressurised for 72 hours then immediately treated with RNA lysis buffer. They were removed from the growth surface using a cell scraper (BD Biosciences, Franklin Lakes, NJ) RNA was extracted with the Stratagene RNA Isolation Kit® (La Jolla, CA). The Promega Reverse Transcription System® (Madison, WI) was used to synthesize cDNA. PCR was performed using the primers and probes from Integrated DNA Technologies, Inc, Coralville, IA and gel electrophoresis was performed in the usual manner.

#### *Quantitative Real Time Polymerase Chain Reaction*

The cDNA derived from cells grown under pressure as described in the previous step was assessed for the relative expression of VEGF-A, VEGF-C and TPA. Standardisation, validation and analysis of the assays have been previously described.<sup>12</sup> Briefly, 5 µL of cDNA were added to 20 µL of separate aliquots of master mix containing primer and probe sequences for VEGF-A, VEGF-C and TPA and the reference house-keeping gene  $\beta$ -actin. Realtime PCR with the iCycler® (Bio-Rad, Hercules, CA) was performed. Cycling parameters were as follows: one 10 minute hot start at 94°C; 45 cycles of 30 second denaturation at 94°C; 1 minute of annealing at 55°C; 30 second extension at 72°C and a final 5 minutes extension at 72°C. These data were normalised to the gene expression of a control known to express VEGF-A, VEGF-C and TPA. We used cDNA from the 143B cell line (ATCC, Manassas, VA) as a control which was treated in the same way and in the same PCR reaction in a 96-well plate.

#### *Immunohistochemistry Staining Procedure*

Immunohistochemistry stains were performed on 10 available biopsy samples taken at the time and site of IFP reading. All immunohistochemistry staining was performed by a single technician in a core facility to ensure standardisation of technique. The antibodies used were CD31 (Catalog number M0823, DakoCytomation, Carpinteria, CA), TPA (Catalog number 387, American Diagnostica Inc, Stamford, CT), VEGF-A (Catalog number sc-152, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and VEGF-C (Catalog number sc-7269, Santa Cruz Biotechnology Inc, Santa Cruz, CA). The controls used were human colonic tissue for CD31 and VEGF-A, human ovarian cancer for VEGF-C and human melanoma for TPA as per the manufacturer recommendation. Primary

antibodies were omitted in a set of samples to serve as a negative control. Immunohistochemical localisation was performed using peroxidase conjugated streptavidin. Slides were deparaffinised in xylene, rehydrated in distilled water, baked at 60°C for 60 minutes and hydrated with distilled water. Pretreatment protocols were used as recommended by the primary antibody manufacturer. Slides were placed in diaminobenzidine (DAB) substrate and the sensitivity sequentially monitored against the respective control. The slides were counterstained using Harris modified haematoxylin (Fisher Scientific, Chicago, IL).

#### *Digital Analysis of Tissue Sections*

The method used was based on the technique described previously.<sup>13</sup> Briefly, slides were surveyed under the an Olympus Vanox-T microscope at 40x magnification and 3 representative fields of each slide were photographed with an Olympus DP12 high-resolution 3.34 megapixel CCD camera (Melville, NY). Images were then processed in Adobe Photoshop LE version 2 (Mountain View, CA). The region of interest was outlined using the magic wand tool and all background areas were converted to black. The images were saved and opened in ImageJ version 1.33 for Windows (NIH, Bethesda, MD). Assessment of vascularity on routine haematoxylin and eosin (H&E) stained slides was done by outlining endothelial lined spaces and analysing the area enclosed by these structures. Assessment of immunohistochemically stained slides was done by analysing the area of the slide stained by the chromagen. The red immunostained area was selected by using the colour threshold command. This was a histogram-based system where 2 peaks were noted – one corresponding to the red-immunostain and one to the blue nucleus. Hue and saturation were adjusted while observing the image in real-time to ascertain if the selection was representative of the tissue section. The percentage composition by surface area of the section was derived from the formula: (Immunostained area/Tissue area) x 100.

#### *Statistical Analysis*

Student's *t*-test was used as a test for significance in continuous variables and the chi-square test was used as a test for significance in categorical variables. Linear regression analysis was used in the assessment of immunohistochemical staining versus the relevant independent variable. Data were captured in a database generated in Microsoft Excel version 10 for Windows NT (Redmond, WA). All statistical analysis was performed using SPSS version 11.5 for Windows NT (Chicago, IL). Data, where relevant, were presented as mean  $\pm$  standard deviation. Unless otherwise specified, error bars on graphs represent the standard error of the mean. Statistical significance was defined as  $P < 0.05$ .

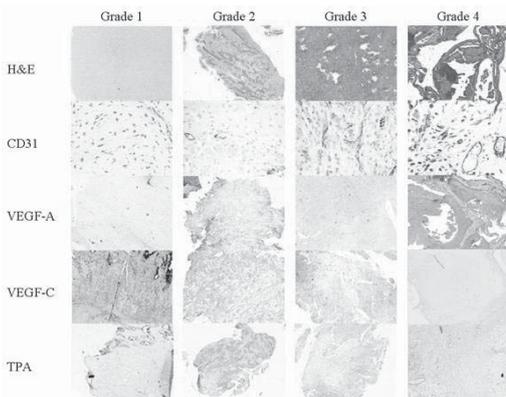


Fig. 1. Vascularity in tumours was assessed using a 4-grade system based on the biopsies made at the time and site of reading of interstitial fluid pressure (40x magnification, H&E). Illustrated here are representative slides in the 4 grades of vascularity seen in the tissue compared with immunohistochemical staining for CD31, VEGF-A, VEGF-C and TPA in the same tissues. There was good correlation between the 4-grade system and ratio of vessel surface area to tumour surface area and there was good correlation between microvessel densities as assessed by CD31 staining and that assessed by H&E staining.

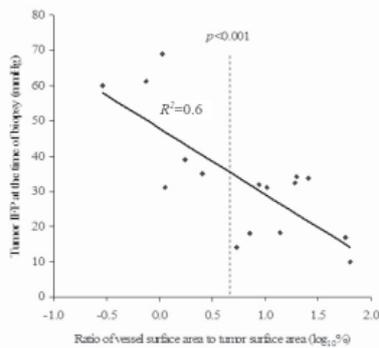


Fig. 3. Vascularity in tumours was inversely related to interstitial fluid pressures ( $R^2=0.6$ ). Mean interstitial fluid pressure in low vascularity tumours was significantly higher ( $P < 0.001$ ) than that in high vascularity tumours (separated by dotted line).

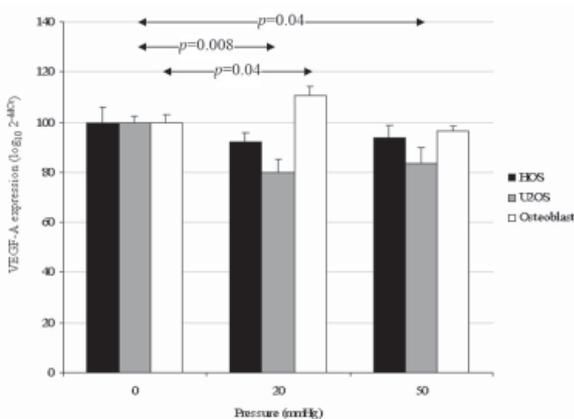


Fig. 5. Quantitative realtime PCR showed that the tumour cell lines (HOS and U2OS) responded to increased ambient pressure by a down-regulation of VEGF-A as opposed to the non-tumour osteoblasts where VEGF-A gene expression was upregulated. Immunohistochemical staining for VEGF-A decreased with increasing interstitial fluid pressure in tumours. Low interstitial fluid pressure tumours had significantly greater ( $P = 0.01$ ) VEGF-A staining than high interstitial fluid pressure tumours.

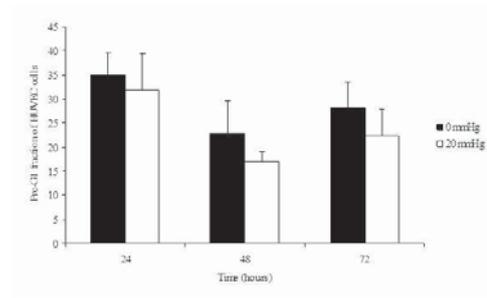


Fig. 2. HUVEC cell growth did not appear to be inhibited in a pressurised environment on microscopic evaluation. On flow cytometry analysis, there was an insignificant increase in proliferative phenotype (cells in S-phase) with pressure and death was not increased in cells grown under pressure. Error bars in all graphs represent the standard deviation of the mean.

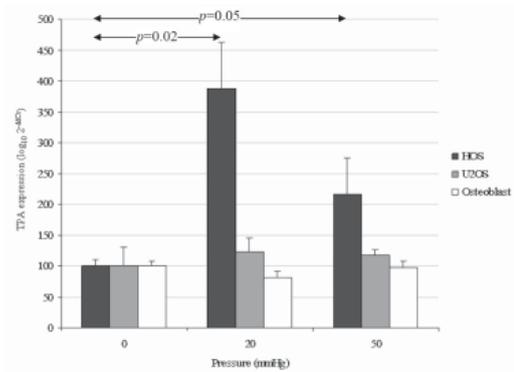


Fig. 4. Quantitative realtime PCR showed that the tumour cell lines (HOS and U2OS) responded to increased ambient pressure by an up-regulation of TPA as opposed to the non-tumour osteoblasts where TPA gene expression was downregulated. Immunohistochemical staining for TPA increased with increasing interstitial fluid pressure in tumours. High interstitial fluid pressure tumours had significantly greater ( $P = 0.0001$ ) TPA staining than low interstitial fluid pressure tumours.

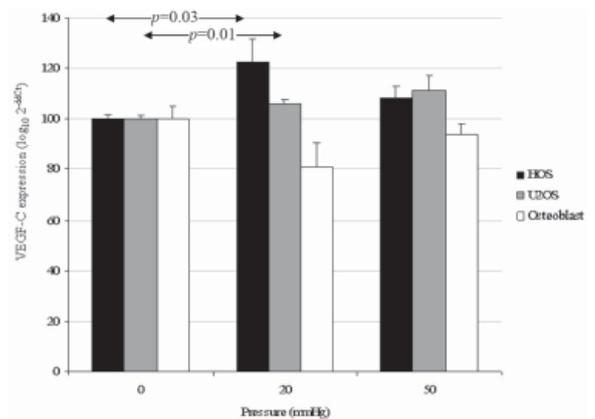


Fig. 6. Quantitative realtime PCR showed that the tumour cell lines (HOS and U2OS) responded to increased ambient pressure by an up-regulation of VEGF-C as opposed to the non-tumour osteoblasts where VEGF-C gene expression was downregulated. Immunohistochemical staining for VEGF-C increased with increasing interstitial fluid pressure in tumours. High interstitial fluid pressure tumours had significantly greater ( $P = 0.008$ ) VEGF-C staining than low interstitial fluid pressure tumours.

## Results

Between January 1997 and June 2000, 16 patients with the diagnosis of high-grade osteosarcoma underwent IFP readings at the time and site of biopsy.

The mean age was  $16.0 \pm 6.7$  years. There were 9 female and 7 male patients. Tumours were in the distal femur in 7 patients, proximal tibia in 5 patients, proximal humerus in 2 patients, distal tibia in 1 patient and sacrum in 1 patient. Tumours had a mean size of  $81.2 \pm 41.2$  cm in the longest dimension. Six tumours were osteoblastic, 3 were chondroblastic, 2 were giant cell rich, 2 were malignant fibrous histiocytoma like, 1 was telangiectatic and 1 was a round cell osteosarcoma. IFP in the osteosarcomas was  $33.5 \pm 17.2$  mmHg, which was significantly greater than that in the normal soft tissue where pressures were  $2.9 \pm 5.7$  mmHg ( $P = 0.00001$ ).

Vascularity in tumours was assessed in the first instance using an observational 4-grade system based on the biopsies made at the time and site of reading of IFP. Each analysed field measured 1700 by 1300  $\mu\text{m}$ . Vessels in grade 1, 2 and 3 tumours measured 20 to 200  $\mu\text{m}$ . Vessels in grade 4 tumours were up to 700  $\mu\text{m}$  in diameter. Ten tumours had high vascularity (grade 3 and 4) and 6 had low vascularity (grade 1 and 2). Grades of vascularity were evenly distributed among all histological sub-types except that telangiectatic osteosarcomas by definition had high vascularity. There was good correlation between the observational grade of vascularity and the percentage composition of the biopsy sample by vessels as quantified on H&E stained slides (Fig. 1). There was good correlation ( $R^2 = 0.82$ ) between microvessel density as assessed by percentage CD31 staining and the percentage composition of the biopsy sample by vessels as assessed on H&E stained slides in the 10 samples available for immunohistochemical staining (Fig. 1). CD31 staining in high vascularity tumours (mean  $5.3 \pm \text{SD } 1.7\%$ ) was significantly greater ( $P < 0.05$ ) than that in low vascularity tumours (mean  $2.3 \pm \text{SD } 2.0\%$ ).

Tumour vascularity was inversely related to IFP ( $R^2 = 0.6$ ) as shown in Figure 3. Six of 11 tumours with high IFP (i.e., greater than 20 mmHg) as opposed to none of 5 tumours with low IFP (i.e., less than 20 mmHg) had low vascularity. Accordingly, the 6 low vascularity tumours had IFPs of  $49.2 \pm 16$  mmHg compared to 10 high vascularity tumours with IFPs of  $24.1 \pm 9.4$  mmHg ( $P = 0.0007$ ). Similarly, CD31 staining in high IFP tumours (mean  $3.4 \pm \text{SD } 1.6\%$ ) was significantly lower ( $P = 0.04$ ) than that in low IFP tumours (mean  $5.9 \pm \text{SD } 1.9\%$ ). There was no significant correlation between vascularity and blood flow in the tumour ( $R^2=0.2$ ).

Raised IFP up to 20 mmHg did not inhibit HUVEC cell growth (Fig. 2). There was no significant difference in the proportion of cells in S phase between HUVEC cells grown

under 20 mmHg pressure versus those grown at 0 mmHg as assessed by cell cycle analysis (Fig. 2). There was no significant increase in death in HUVEC cells grown under 20 mmHg versus those grown at 0 mmHg as assessed by the pre-G1 fraction in cell cycle analysis (Fig. 2).

Qualitative PCR showed that there was high expression of TPA, VEGF-A and VEGF-C in all osteosarcoma cell lines. In the osteoblast cell line, the expression of all markers was lower than that in osteosarcoma cell lines (data not shown).

In osteosarcoma cells grown under pressure, TPA expression was upregulated compared to osteoblasts grown under pressure by quantitative PCR (Fig. 4). This phenomenon was most pronounced at 20 mmHg. Growth of HOS at 20 mmHg and 50 mmHg was associated with a statistically significant upregulation of TPA compared with growth at 0 mmHg ( $P = 0.02$  and  $P = 0.05$  respectively). By comparison, in osteoblasts, there was a downregulation of TPA with pressure. Immunohistochemistry validated this observation. TPA staining on biopsy samples increased progressively with increasing tumour IFP. High IFP tumours had significantly greater TPA staining than low IFP tumours ( $P = 0.0001$ ).

VEGF-A expression was downregulated in osteosarcoma cells grown under pressure compared to osteoblasts where VEGF-A expression was relatively unaffected by increasing IFP on quantitative PCR (Fig. 5). In particular growth of U2OS at 20 mmHg and 50 mmHg was associated with a statistically significant down-regulation of VEGF-A ( $P = 0.008$  and  $P = 0.04$ ) compared to growth of U2OS at 0 mmHg. In addition, growth of osteoblasts at 20 mmHg was associated with a statistically significant upregulation of VEGF-A ( $P = 0.04$ ) compared to growth of osteoblasts at 0 mmHg. Low IFP tumours had significantly greater VEGF-A staining than high IFP tumours ( $P = 0.01$ ).

VEGF-C expression was upregulated in osteosarcoma cells grown under pressure whereas in osteoblasts, VEGF-C expression was downregulated in cells grown under raised pressure by quantitative PCR. In particular, growth of HOS and U2OS at 20 mmHg was associated with a significant upregulation ( $P = 0.03$  and  $P = 0.01$ ) of VEGF-C compared to growth of these cells at 0 mmHg (Fig. 6). High IFP tumours had significantly greater VEGF-C staining than low IFP tumours ( $P = 0.008$ ).

## Discussion

In this study, we suggest that vascularity in osteosarcomas may be reduced by constitutively raised IFP. The study is limited by the fact that there were only 16 patients in the study. Based on the blinded 4-part observational score by the senior pathologist in the study, tumours with high IFP were found to have low vascularity and those with low IFP were found to have high vascularity. This was further

supported by image analysis quantification of the amount of vascularity in these tumours. Tissue blocks were available in 10 patients for immunohistochemical assessment, which confirmed our observation.

The reduction in vascularity with increased IFP was not due to the inhibition of growth of endothelial cells in a pressurised environment. Instead, by isolating the osteosarcoma cells in a cell culture system that permits cells growth in a physiologically replicated high-pressure environment, we were able to demonstrate that there was a statistically significant down-regulation of angiogenic factors (VEGF-A), up-regulation of anti-angiogenic factors (TPA) and up-regulation of factors associated with lymphangiogenesis (VEGF-C) on PCR.<sup>5,6,10</sup> Immunohistochemical analysis for angiogenic factors in biopsy samples taken at the time and site of IFP readings seem to corroborate PCR findings. While IFP in the *in vivo* tumour has some minute to minute variation presumably due to blood flow and respiration, this variation is small and hence static pressure was used in our model.

While statistically significant ( $P < 0.05$ ), quantitative PCR findings which differed from controls by less than 20% may not have biological significance. Ideally, quantitative protein analysis of tissue samples using ELISA or Western blot is desirable but requires fresh tissue samples, which were not available in this study. Similarly, our pressurised cell culture environment does not permit accurate assessment of protein levels in the supernatant. Furthermore, we only used 2 osteosarcoma cell lines and hence there is a possibility that these findings are cell line specific. These data should therefore be considered preliminary. Studies are presently underway using an animal model to address these shortcomings.

The response to VEGF-A has implications on the use of anti-VEGF-A therapy and provides a plausible mechanism for suboptimal response to anti-angiogenic therapy. We hypothesize that high-pressure tumours with low levels of VEGF-A expression would be poor candidates for anti-angiogenesis therapy. Furthermore, TPA, also being upregulated in high-pressure tumours, has an indirectly inhibitory effect on VEGF-A production through the formation of angiostatin. These tumours with low levels of vascularity and angiogenesis would therefore be predicted to be poorly responsive to anti-angiogenic factors.

This finding may have implications on drug delivery to the tumour. It has been shown that high-pressure tumours do not necessarily have reduced blood flow as per a traditional Starling resistor model of flow.<sup>14</sup> Solid tumour models for

instance have capillary distension pressures in post-capillary venules equivalent to that of their IFPs.<sup>11</sup> Hence, raised IFP could not have reduced flow in these tumours, as the pressure in the post capillary venules would be less than that of the IFP if that were the case. Nevertheless, these tumours do have low vascularity and hence may explain some reports which have shown that high-pressure tumours have low blood flow (i.e., the low perfusion in tumours with high-pressure may be primarily due to the reduced vascularity in these tumours rather than the high pressure impeding blood flow per se).<sup>2</sup>

It has recently been reported that tumours elaborate high levels of VEGF-C in their depths although functioning lymphatics are present only on the surface of tumours.<sup>8</sup> These findings suggest that solid tumours metastasize via the lymphatics on the surface of tumours. We acknowledge that osteosarcoma is a poor model to demonstrate lymphatic metastasis. Nevertheless, our finding that this solid tumour has a pressure response element that drives VEGF-C production identifies a possible mechanism that could be explored in solid tumours that may influence metastasis. This abortive physiological response to elevated IFP appears to be aimed at reducing IFP through lymphatic formation. It is interesting to note that in all gene-expression experiments the osteoblasts responded in a reverse manner to the elevated fluid pressure from the tumours (i.e., TPA and VEGF-C were downregulated with pressure and VEGF-A was upregulated).

Raised IFP in tumours occurs due to leakage of proteins through permeative microvasculature inherent to most tumours.<sup>7</sup> Tumour growth would naturally result in increased angiogenesis to support itself. Our previous study has shown that the raised IFP in tumours can result in increased growth setting up a vicious cycle.<sup>1</sup> In light of the present study, we would now investigate the presence of an innate pressure-sensing mechanism to IFP in osteosarcomas. This may have the effect of countering the vascularity in these tumours thus reducing the IFP both directly (via inhibition of VEGF-A) and indirectly through an abortive attempt at forming lymphatics. Lymphatics on the surface of the tumours, however, are not inhibited as has been previously shown<sup>8</sup> and hence the rise in IFP may drive this surface lymphangiogenesis.

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