Supernatants derived from chemotherapy-treated cancer cell lines can modify angiogenesis

WM Liu*,1, JL Dennis2, AM Gravett1, C Chanthirakumar1, E Kaminska1, G Coulton2, DW Fowler1, M Bodman-Smith1 and AG Dalgleish1

1Department of Oncology, Division of Clinical Sciences, St George’s, University of London, 2nd Floor, Jenner Wing, London SW17 0RE, UK; 2Medical Biomics Centre, Division of Basic Molecular Sciences, St. George’s, University of London, London, UK.

BACKGROUND: There is evidence that tumours produce substances such as cytokines and microvesicular bodies bearing bioactive molecules, which support the carcinogenic process. Furthermore, chemotherapy has also been shown to modify these exudates and in doing so, neutralise their tumourigenic influence.

METHODS: In the current study, we have investigated the effect of chemotherapy agents on modifying the cytokine profile and microvesicular cargo of supernatants derived from cancer cell lines. In addition, we have explored the effect of these tumour-derived supernatants on angiogenesis, and how chemotherapy can alter the supernatants rendering them less pro-angiogenic.

RESULTS: Herein, we show that supernatants contain a rich cocktail of cytokines, a number of which are potent modulators of angiogenesis. They also contain microvesicular bodies containing RNA transcripts that code for proteins involved in transcription, immune modulation and angiogenesis. These supernatants altered intracellular signalling molecules in endothelial cells and significantly enhanced their tubulogenic character; however, this was severely compromised when supernatants from tumours treated with chemotherapy was used instead.

CONCLUSION: This study suggests tumour exudates and bioactive material from tumours can influence cellular functions, and that treatment with some chemotherapy can serve to negate these pro-tumourigenic processes.

Keywords: tumour-supernatants; chemotherapy; immunotherapy; angiogenesis; immune-modulation; microvesicles

We have previously shown that some chemotherapies are able to affect immune modulation (Liu et al, 2010). This is manifest in two ways, first chemotherapy can upregulate class I human leucocyte antigen expression on tumours directly, which leads to an improved adaptive response. Second, an indirect effect of chemotherapy is possible as supernatant derived from tumours treated with standard drugs such as oxaliplatin (OXP) or gemcitabine can enhance the function of dendritic cells (DCs). Specifically, DCs exposed to these supernatants have an enhanced maturation phenotype, which in turn increases the activation and proliferation of T-lymphocytes, and ultimately the cytolytic ability of these effector cells. The effects on DCs were apparent at the level of gene expression, with the supernatants being capable of modifying the expressions of DC markers such as CCR7, CD80 and CD86 (Liu et al, 2012).

It appears that chemotherapy can hijack tumours resulting in their producing substances that are anti-tumour in character. Moreover, as tumours seem to intrinsically produce these substances, chemotherapy may actually serve to negate/neutralise the action of tumour-derived supernatants. Indeed, the presence of a bridge of communication between tumours and the host microenvironment suggests its role in the tumourigenic process (Joyce and Pollard, 2009). Communication can be direct through tumour-to-stroma contact, or via an indirect mechanism such as supernatants drawn from tumours. These supernatants invariably reflect the cellular and biochemical makeup of the tumour, and can exhibit a repertoire of cytokines (Dranoff, 2004). Their components appear to support tumourigenesis, but the particular roles they have in the process are unclear. However, we have shown that tumour-derived cytokines can support angiogenesis (Liu et al, 2009), and directly maintain an immunosuppressive microenvironment conducive to cancer development (Dalgleish and O’Byrne, 2002).

Supernatants can also include cellular fragments, apoptotic bodies and other plasma membrane-derived vesicles. These microvesicular structures have been detected in a number of biological fluids including blood and ascitic fluid, and are thought to be involved in communication between cells, including cross talk between tumour cells and host (Thery et al, 2002). In a similar way to cytokines, microvesicles derived from some tumours may have a part in suppressing immune responses directed towards the cancer, as well as modifying the tumour microenvironment to support tumourigenesis. Although the exact mechanism by which this extracellular communication is achieved is unknown, RNA and microRNA material have been found within the vesicles as well as adhered to these microparticles, thus rendering them translatable protagonists (Valadi et al, 2007; Pegtel et al, 2010). The resulting effects have included increased metastatic potential and modulation of angiogenesis (Hood et al, 2011; Taverna et al, 2011).
MATERIALS AND METHODS

Cell culture

The human cancer cell lines A549 (lung), HCT116 (colon), MCF7 (breast) and human umbilical vein endothelial cells (HUVECs) were obtained from the Cancer Research UK Cell Production Laboratories (Potters Bar, UK). All cells were maintained in culture medium supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (basal culture medium). All cell lines were incubated in a humidified atmosphere with 5% CO₂ in air at 37°C, and cancer cell lines were discarded when the passage number exceeded 15.

Reagents

Cyclophosphamide (CPM: Sigma Ltd., Dorset, UK) and OXP (Sigma) were dissolved in phosphate buffered saline (PBS) to create 10 mM stock solutions that were maintained at −20°C for no longer than 4 weeks. All controls used in our studies involved treatment with equal amounts of PBS.

Generating tumour-derived supernatants

Supernatants were decanted from these cultures as described previously (Liu et al., 2012). Briefly, exhausted media (supernatants) were obtained from cells either cultured alone, with 100 μM CPM or with 1 μM OXP for 72 h. These concentrations were the approximate IC25s for the drugs as reported previously (Liu et al., 2010). All supernatants were stored at −20°C, and freeze–thaw cycles kept to a minimum by aliquoting.

Cytokine analysis of supernatants

The profile of 46 cytokines and analytes associated with inflammation were determined in each of the supernatants by a proprietary multiplex immunoassay solution (Rules Based Medicine Inc., Austin, TX, USA). In addition, the levels of VEGF were assayed using an ELISA kit according to the manufacturer’s instructions (R&D Systems, Oxford, UK).

Isolation of microvesicles from supernatants

Small cellular vesicles and microparticles were isolated by sequential centrifugation at 4°C using increasing forces. These were loosely defined as microvesicles. Culture supernatants were initially centrifuged at 300 g for 10 min, and then transferred to a fresh tube for a second spin at 2000 g for 10 min. These steps allowed for the separation of live and dead cells, respectively. Supernatants were then centrifuged for a third time at 10 000 g for 30 min as a way of removing cellular debris. The supernatants were then transferred to fresh tubes, and ultra-centrifuged at 100 000 g for 70 min, before transferring the supernatants to a fresh tube. These samples were defined as the ‘cytokine fraction’. The remaining pellets were then re-suspended in PBS. A final spin at 100 000 g for 70 min was performed before supernatants were aspirated, yielding pellets of microvesicles that were stored at −80°C until required.

RNA extraction

RNA was extracted from microvesicles collected from the supernatants of A549 cells cultured alone, with CPM or with OXP. RNA was purified by Trizol, followed by precipitation with iso-propanol. The RNA pellet was washed in 70% (v/v) ethanol, air dried, re-suspended in RNase-free water and stored at −80°C. RNA concentration and purity were measured using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was determined by an Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd., Stockport, UK) using RNA 6000 Nano LabChips (Agilent). RNA integrity was expressed in terms of an RNA integrity number as determined by the proprietary software and only those with values of > 9.0 were progressed.

Illumina microarrays

Biotinylated cRNA was generated from 100ng total RNA using the Illumina Total Prep RNA Amplification Kit (Applied Biosystems, Warrington, UK) according to manufacturer’s instructions. The concentration and purity of resultant cRNA was determined using the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies). Equal amounts (750ng) of cRNA were hybridised to the Illumina human HT12-v3 arrays (Illumina UK, Saffron Walden, UK) for 18h and subsequently processed according to manufacturer’s instructions before scanning on an Illumina Bead Array Reader (Illumina UK). The image data were processed using default values in Genome Studio v2009.1 (Illumina, UK), before loading onto Gene Spring v9.0 (Agilent Technologies UK Ltd.) for data normalisation and filtering. Analyses were performed using gene ontology databases within Pathway Studio v7.1 (Ariadne Genomics, Rockville, MD, USA). A greater than 0.25-fold change was used as our cutoff magnitude for gene list compositions by using Excel software (Microsoft UK, Reading, UK).

Tubule-formation assay

The vasculogenic natures of supernatants, microvesicles and cytokine fractions were assessed by their ability to induce the reorganisation on HUVECs into primitive tubules. A layer (150 μl) of growth factor reduced Matrigel matrix was allowed to set in six-well plates for 30 min, before laying on HUVECs (5 × 10^4 in medium). This medium was either (i) complete supernatants (500 μl) derived from A549 cells cultured with chemotherapy; or (ii) isolated microvesicles re-suspended at a protein concentration of 10 μg per 500 μl in PBS; or (iii) cytokine fraction (500 μl). Samples were incubated in a humidified atmosphere with 5% CO₂ in air at 37°C for 16 h, before visualising tubules under light microscopy. Tubule formation was quantified by using the programme AngioQuant (http://www.cs.tut.fi/sgn/csb/angioquant/).

Immunoblotting analysis

Cells were harvested and total cellular protein was solubilised in lysis buffer (New England Biolabs, Hitchin, UK) and resolved by tris-glycine electrophoresis using a 4–12% bis-tris gradient gel. Following transfer of proteins to 0.45 μm nitrocellulose membranes, blocking was performed in 5% (w/v) non-fat milk in TTBS (0.5% (v/v) Tween-20 in tris buffered saline (50 mM Tris base with 150 mM NaCl; pH 7.6)). Primary antibody probing was performed with anti-AKT, anti-phospho AKT, anti-ERK and anti-phospho ERK. All primary antibodies were obtained from New England Biolabs and used at a dilution of 1:1000, unless stated otherwise. Anti-β actin was used as a loading control (1:2000 – New England Biolabs). Following three washing steps in TTBS, horseradish...
peroxidase-conjugated anti-species IgG was used as the secondary antibody (Amersham Biosciences Ltd., Little Chalfont, UK). Bands were visualised by the ECL-plus detection system (Amersham).

RESULTS

Tumour-derived supernatants contain cytokines

The profiles of cytokines in the supernatants derived from cell lines were assessed by a multiplex immunoassay, where 46 analytes associated with inflammation were quantified. The effect of treating cells with equi-active concentrations of CPM or OXP on cytokine profiles was also established. Only those analytes that were detectable are presented. Results indicated that the supernatants contained variable amounts of cytokines, which were different in the cell line studies (Figures 1A–C). Specifically, HCT116 cells produced supernatants that consisted of 13 cytokines, compared with 6 and 4 for A549 and MCF7, respectively; and there were no cytokines that were common to all three tumours. Culturing cells with CPM did not affect the levels of these cytokines in the cells; however, there was a divergence in the levels of the cytokines after treatment with OXP. Cytokines were thus ordered according to the difference in amounts between untreated and OXP-treated samples, and VEGF was seen to be altered to the greatest extent. The amount of VEGF in the supernatants were then re-assessed by ELISA, and results showed that they were significantly decreased after treatment with OXP in A549 (1.1 ± 0.55 pg ml⁻¹ vs 36 ± 2.4 pg ml⁻¹ in untreated controls) and in HCT116 (571 ± 29 pg ml⁻¹ vs 1741 ± 48 pg ml⁻¹ in untreated controls) (Both P<0.001 Figure 1D).

Tumour-derived supernatants can support vasculogenesis

We next tested the vasculogenic nature of supernatants by culturing them with HUVECs and assessing the extent to which they re-arranged into primitive tubules when grown upon Matrigel. The supernatants were deconstructed by differential ultra-centrifugation into microvesicular and cytokine fractions. The protein contents of the microvesicular fractions were assessed by a proprietary bicinchoninic acid assay kit (Fisher Scientific UK Ltd., Loughborough, UK) (Figure 2A), and then used at a concentration of 10 μg per 500 μl in PBS. There was thus a total of three samples assessed: (1) complete supernatant, (2) microvesicular fraction and (3) cytokine fraction. Results showed that culturing HUVECs with standard culture medium or with basal medium spiked with 100 μM of CPM or 1 μM of OXP.
of OXP for 72 h, resulted in no tube formation (Figure 3). However, culturing with CONT-supernatant significantly increased the capacity of HUVECs to form tube-like structures. This was enumerated by measuring the total lengths of tubule-complexes by using the dedicated image analysis tool AngioQuant, which reported an increase from 0 to 2806 ± 225 a.u. (Figure 3).

Furthermore, all cells were confirmed viable by trypan blue exclusion analysis. CPM-supernatants also resulted in a similar extent of tubulogenesis (paired \( t \)-test vs controls: \( P = 0.161 \)); however, OXP-supernatant significantly reduced the level of tube formation (1362 ± 192 vs 2806 ± 225 in CONT-supernatant; \( P = 0.006 \)). The trend in tubulogenesis following stimulation with cytokine fractions was similar to that seen with complete supernatants, in that the level of network formation was high in CONT and CPM samples, but significantly reduced in OXP samples (961 ± 57 vs 2193 ± 211; \( P = 0.003 \)). HUVECs stimulated with microvesicles generally resulted in fewer tubes as compared with the number seen with complete supernatants. Specifically, although microvesicles from untreated tumours significantly increased tubule formation, the amount was much lower than that induced by complete medium (842 ± 91 vs 2806 ± 225; \( P < 0.001 \)) (Figure 3). There was no difference in the tubulogenic nature of microvesicles from CPM-treated and untreated tumours (\( P = 0.886 \)). Conversely, those from OXP-treated tumours had slightly increased tubulogenic capability, which did not reach significance (\( P = 0.064 \)) (Figure 3).

Tumour-derived supernatants alter the expressions of AKT and ERK

To investigate whether the changes in vasculogenesis were linked with modifications to key intracellular signalling pathways, whole-cell lysates were obtained from HUVECs cultured with the supernatants and the levels of AKT and ERK assessed by immunoblotting. This panel was chosen as they broadly represented key proteins involved in modifying angiogenesis and neovascularisation. The intention was to employ this approach to identify whether or not a change in intracellular signalling was involved in triggering the phenotypic changes, which could ultimately provide putative targets against which therapies could be adapted. Results highlighted increases in active AKT and ERK proteins following culture with CONT-supernatants and CPM-supernatants (Figure 2B). However, these increases were not seen in HUVECs cultured with OXP-supernatant, and their levels remained virtually unchanged compared with HUVECs in basal medium (Figure 2B).

Sample-descriptions, microarray data quality control, filtering and pre-processing

There were two independent sets of microvesicles harvested from A549 tumour cells in separate experiments, and each set comprised of three treatment conditions: (i) untreated; (ii) CPM-treated and
(iii) OXP-treated, from which RNA was extracted. The qualities of the RNA from each of the samples were confirmed by Illumina software (Illumina), and all controls (hybridisations, negative, spike-ins, etc) were within the guidelines as specified by the manufacturer. All pre-normalised intensity signals from each probe were collated and those genes whose expressions were below the baseline/background value of 60 were defined as absent. Additionally, those probes with no names or those designated ‘predicted genes’ were also excluded. This filtering process trimmed the number of genes from 45 599 to 28 557. Differences between CPM and untreated samples in the magnitudes of gene expressions between any of the treatment groups were then analysed using Excel software.

Comparing the transcriptomes of microvesicles

An initial survey of the genes showed there to be a very small number present in the microvesicles. For instance, there were just 92 hits from a possible 28 557 genes (0.32%) in the microvesicles from the supernatants of untreated tumour cells. Full gene lists are available at GeneExpress (www.ebi.ac.uk – accession number E-TABM-1201). A large proportion of these genes possessed similar functions, so gene ontological analysis using Pathway Studio was performed on this list. Categories were then defined and ranked in order of the percentage of genes found. The leading categories were regulation of transcription (15% (12/92)), and genes for membrane components (27% (25/92)) (Table 1). The expressions of these 92 genes were then established in the microvesicles harvested from tumours cultured with CPM or OXP (Table 1). Venn analysis of all the transcripts called present in the microvesicles from any of the treatments showed a proportion of them was common (Figure 2C). There were overlapping genes unique to CPM or OXP with the untreated sample (28 and 14, respectively), but few between CPM and OXP (3) (Figure 2C).

In an attempt to understand the genetic backdrop of the general observation that the vasculogenic action of microvesicles were not different between CPM and untreated samples but different in OXP-treatment, a second list of genes were created that complied with the criteria that expressions be (i) <10% different between untreated and CPM-treated, and (ii) >1-fold change in either direction between untreated and OXP-treated (Table 2). Furthermore, this approach was also in accordance with our previous published data that indicated CPM generally affected cells to a lesser degree than OXP (Liu et al, 2010, 2012). Analysis revealed just 13 genes (0.046%) conformed to these criteria, and that a number of them (il18, lilrb1, krit1, abca4 and map1a) were associated with angiogenesis and neovascularisation.

DISCUSSION

This study was initiated in response to our earlier reports defining a novel potential immune-stimulatory feature of some common chemotherapy agents. In these studies, we showed an enhancement to cell-mediated immune responses by supernatants derived from tumour cells, and that these were affected by drug treatments (Liu et al, 2010). Therefore, the aim of the current study was to explore the biological nature of these supernatants. To this end, we deconstructed the supernatants into cytokine and microvesicular fractions, and employed molecular biological techniques to establish the effect on cellular processes of the individual contributions of these parts. Our results showed that the supernatants were rich in cytokines with the capacity to alter angiogenesis, as well as containing microvesicles loaded with RNA material. These supernatants altered intracellular signalling, reorganised the phenotype of HUVECs, and thus modified the process of tubulogenesis. Importantly, our data also supported the idea that some chemotherapy could negate these effects, and hence minimise the impact of these neoplastic events.

Cancer cells produce a heterogeneous mixture of material that has been loosely referred to as debris, apoptotic fragments or microvesicular matter. Rather than being haphazard and inert in nature, they have been shown to be organised into vesicles filled with bioactive material (Thery et al, 2002). Their presence in biological fluids such as blood, urine and ascites supports their role in cell-to-cell communication, and suggests an involvement in the carcinogenic process (Zhang and Grizzle, 2011).
The overlapping association of these microvesicles with soluble factors such as cytokines, which together are constantly exuded by tumours generates a potent mixture that is capable of transmitting developmental cues between tumour and host cells. It would be valuable to identify the key elements in the supernatants as this would help us understand our earlier data. For that reason, we assessed the cytokine fraction of the supernatants using a multiplex immunoassay that simultaneously quantified 46 analytes related to inflammation. Results showed that the cytokines identified in the supernatants were varied and tumour specific. The specific roles and impacts of these analytes were not assessed; however, it appeared that a number of these were associated with angiogenesis. In particular, there were a number of analytes with strong links to neo-vascularisation that were detected in the supernatants of untreated HCT116 cells, and of these, we focused primarily on VEGF, the cytokine that was altered the greatest.

In addition to our initial multi-analyte profiling approach, we also separated the microvesicular fraction from the soluble and debris fractions of the supernatants by differential ultra-centrifugation. Technically, this population of microvesicles would have been mixed with those shed by FBS (Heijnen et al, 1999). However, as FBS was present in all our supernatants, any changes to their profile would be negated or minimise their impacts. Not only that, this would also help us understand our earlier data. For that reason, we assessed the cytokine fraction of the supernatants by using a multiplex immunoassay that simultaneously quantified 46 analytes related to inflammation. Results showed that the cytokines identified in the supernatants were varied and tumour specific. The specific roles and impacts of these analytes were not assessed; however, it appeared that a number of these were associated with angiogenesis. In particular, there were a number of analytes with strong links to neo-vascularisation that were detected in the supernatants of untreated HCT116 cells, and of these, we focused primarily on VEGF, the cytokine that was altered the greatest.

In addition to our initial multi-analyte profiling approach, we also separated the microvesicular fraction from the soluble and debris fractions of the supernatants by differential ultra-centrifugation (Thery et al, 2006), and assessed the genetic cargo of these membrane bound particles by microarray analysis. Technically, this population of microvesicles would have been mixed with those shed by FBS (Heijnen et al, 1999). However, as FBS was present in all our supernatants, any changes to their profile would be
automatically factored in. Our initial intention was to use this methodology as a way of first, establishing if any the presence of RNA transcripts; and second, to identify those that were found. We intentionally did not treat these microvesicles with RNAs to remove surface RNA, as physiologically, these may also have a putative role in communication. Additionally, it was not our focus to discriminate the individual components of the microvesicles (Simpson et al., 2008), and thus treated them as one entity. Results of the gene expression analysis revealed just 92 genes out of a possible 28557 genes (0.32%) were found in the microvesicles. A number of these genes were primarily associated with transcription and immune function, which reinforced our initial studies that suggested tumour-derived supernatants were exploitable targets for chemotheraphy with regards to enhancing immunosurveillance and immune function (Liu et al., 2010, 2012). Interestingly, the results of this analysis also identified a cohort of genes specifically involved in angiogenesis was also represented in the microvesicles.

To address the suggestion that supernatants were able to influence angiogenesis, we next assessed the ability of tumour supernatants to re-organise HUVECs into primitive tube-structures on Matrigel. We first demonstrated an increased active state of intracellular molecules ERK and AKT in HUVECs, which are important protagonists intimately associated with the angiogenesis (Carmeliet and Jain, 2011), following treatment with supernatants from tumours. The phenotypic consequence of this was significant increases in tube formation by HUVECs. This is not only seen in complete supernatants, but also seen when HUVECs were stimulated specifically with the cytokine fraction of deconstructed supernatant. This was a possible reflection of the pro-angiogenic factors detected by the immunoassays. Moreover, a primitive supernatant. This was a possible reflection of the pro-angiogenic factors detected by the immunoassays. Additionally, it was not our focus to discriminate the individual components of the microvesicles. Furthermore, the results of this analysis also identified a cohort of genes specifically involved in angiogenesis was also represented in the microvesicles.

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REFERENCES

Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473: 298 – 307
Dalgleish AG, O’Byrne KJ (2002) Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer. Adv Cancer Res 84: 231–276
Dranoff G (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 4: 11 – 22
Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood 94: 3791 – 3799
Hood JL, San RS, Wickline SA (2011) Exosomes released by melanoma cells prepare sentinel lymph nodes for tumour metastasis. Cancer Res 71: 3792 – 3801
Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9: 239 – 252
Liu WM, DeNise JL, Fowler DW, Dalgleish AG (2012) The gene expression profile of unstimulated dendritic cells can be used as a predictor of function. Int J Cancer 130: 979 – 990
Liu WM, Fowler DW, Gravett AM, Smith P, Dalgleish AG (2011) Supernatants from lymphocytes stimulated with Bacillus Calmette-Guerin can modify the antigenicity of tumours and stimulate alloimmune T-cell responses. Br J Cancer 105: 687 – 693

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Liu WM, Fowler DW, Smith P, Dalgleish AG (2010) Pre-treatment with chemotherapy can enhance the antigenicity and immunogenicity of tumours by promoting adaptive immune responses. Br J Cancer 102: 115–123
Liu WM, Henry JY, Meyer B, Bartlett JB, Dalgleish AG, Galustian C (2009) Inhibition of metastatic potential in colorectal carcinoma in vivo and in vitro using immunomodulatory drugs (IMiDs). Br J Cancer 101: 803–812
Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Würdinger T, Middeldorp JM (2010) Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci USA 107: 6328–6333
Simpson RJ, Jensen SS, Lim JW (2008) Proteomic profiling of exosomes: current perspectives. Proteomics 8: 4083–4099

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