Angiogenesis consists a fundamental mechanism required for a number of pathophysiological processes, among others tumour growth. It constitutes a dynamic process and a high number of proangiogenic and antiangiogenic molecules have critical roles in regulating angiogenesis. In response to stimuli, such as hypoxia, oxidative stress and acidosis, several proangiogenic factors are released from endothelial, stromal and tumour cells into the microenvironment (Gasparini et al, 2005). Over 30 angiogenic factors have been identified and reported to have important roles in angiogenesis, with the dominant growth factor controlling this process being the vascular endothelial growth factor (VEGF) (Ribatti and Vacc, 2008). Vascular endothelial growth factor is a dimeric polypeptide with molecular weight ranging from 35 to 45 kDa. Vascular endothelial growth factor refers to a family of growth factors, comprising seven different proteins, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placent growth factor isoforms 1 and 2 (Lyons et al, 2010). Direct actions of VEGF include stimulation of endothelial mitogenesis, promotion of endothelial survival/control of vascular permeability and promotion of cell migration (Zucker et al, 1998; Tam et al, 2006).

Moreover, it has been recently proposed that VEGF may have a direct effect on tumour cell growth (Tam et al, 2006). Vascular endothelial growth factor molecules exert their angiogenic effects by binding to specific VEGF receptors (VEGFR). Vascular endothelial growth factor ligands bind to three primary receptors, namely VEGFR-1 and VEGFR-2 associated with angiogenesis, and VEGFR-3, which is associated with lymphangio-genesis (Zucker et al, 1998; Ferrara, 2004; Tam et al, 2006; Lyons et al, 2010; Waldner et al, 2010). Vascular endothelial growth factor receptor-2 (Flk-1/KDR) is responsible for the majority of the downstream angiogenic effects of VEGF, including microvascular permeability, endothelial cell proliferation, invasion, migration and survival (Bergers and Benjamin, 2003). Recent data suggest that VEGF contributes to tumour growth not only by promoting angiogenesis but also by an indirect mechanism of regulation of T cells involved in the immune response against the tumour (Bergers and Benjamin, 2003; Hicklin and Ellis, 2005; Mimura et al, 2007; Zindl and Chaplin, 2010). More specifically, VEGF has been demonstrated in vitro to hinder the functional maturation of dendritic cells from their hematopoietic progenitors (Gabrilovich et al, 1996). In addition, VEGF, at pathophysiological concentrations, interferes with the thymic development of T cells from early hematopoietic progenitor cells (Ohm et al, 2003). T cells are pivotal in the induction and progression of the anti-tumour immune reaction in many types of cancer, including ovarian cancer.
carcinomas and cancer patients demonstrate impaired T-cell functions; however, the direct effect of VEGF on activation and proliferation of T cells present in ovarian cancer patients' ascites and the means via which this effect is mediated, have not been addressed.

We have previously demonstrated that the levels of VEGF in the ascitic fluid was an independent adverse prognostic factor in a cohort of 65 patients with advanced ovarian cancer and were inversely correlated with immunologically important T-cell subpopulations, such as CD3^+^CD56^+^ NK-like T cells (Bamias et al., 2008). These results were indicative of an additional immunosuppressive function exerted by this factor. We have also demonstrated that VEGF directly suppresses the activation of peripheral blood T cells from ovarian cancer patients and healthy individuals, and that this effect is exerted specifically via VEGFR-2 (Ziogas et al., 2012). Nevertheless, the concentration of VEGF necessary to suppress immune cell functions in vitro was considerably higher than that determined in the blood of ovarian cancer patients and rather resembled VEGF levels recorded in their ascites (Ziogas et al., 2012). This indicates that VEGF-mediated immunosuppression is more likely to occur in the peritoneal cavity, which is an environment particularly enriched in cancer cells.

Based on the aforementioned background, we studied the possible direct effect of VEGF on T cells isolated from the ascites of ovarian cancer patients. We also studied the expression of VEGFRs on these T cells and the effect of VEGF on their cytokotoxic activity against cancer cell lines and autologous cancer cells.

**MATERIALS AND METHODS**

**Cell isolation and culture**

The study protocol had the appropriate IRB approval and informed consent was given by all subjects for the collection of ascitic fluids. No patient had undergone chemotherapy before sample collection. Ascitic fluids were collected from ovarian cancer patients in heparinised tubes via abdominal paracentesis as described (Bamias et al., 2007) and centrifuged to pellet tumour and mononuclear cells. Separation of tumour cells from the mononuclear cells was performed by centrifugation on 75–100% discontinuous Ficoll-Histopaque (Sigma Chemical Co., St Louis, MO, USA) density gradient for 30 min at 400 g at 16 °C. Layers of tumour and mononuclear cells were collected and washed twice in Hank's Balanced Salt Solution (GIBCO BRL, Grand Island, NY, USA). Cells were cultured as previously described (Ziogas et al., 2012). All samples processed, had cell viability over 80%. Tumour cells were cryopreserved in 90% fetal bovine serum (GIBCO BRL), 10% dimethyl-sulfoxide (Sigma Chemical Co.), Mononuclear cells were resuspended in culture medium consisting of RPMI 1640 Glutamax (GIBCO BRL), supplemented with 10% fetal bovine serum, 50 μg ml⁻¹ garamycin (GIBCO BRL) and 10 μg Hepes (GIBCO BRL), at a final concentration of 5 × 10⁶ cells per ml and incubated for 2 h at 37 °C, 5% CO₂ in 100-cm² tissue culture plates (Greiner Bio-one). After 48 h, the medium was changed and cells were incubated for 3 days with 150 IU ml⁻¹ rVEGF (R&D Systems) and rhIL-2 every 3–4 days, for a total of 14 days. To study the dose-dependent effect of VEGF on T-cell proliferation, rVEGF (R&D Systems) was added in cultures at 3–4 day intervals, at the concentrations indicated in the respective figures. Each VEGF concentration was tested in triplicate wells plated with the same T cells and mean values were used for analyses. Cultures were incubated at 37 °C, 5% CO₂. Cell expansion in cultures was assessed using an improved Neubauer hemocytometer. Cell proliferation was tested using the 3H-thymidine incorporation assay according to Ziogas et al (2012) and Georgaki et al (2009). To block VEGFR-2 activity, 1 μg ml⁻¹ of neutralising anti-VEGFR-2 mAb (R&D Systems) was added in cultures on days 0, 1, 3 and consequently every 3 days.

**Flow cytometric analysis**

Cultured T cells were analysed for surface expression of VEGFRs on days 0, 7 and 14, using fluorescence activated cell scanning, as previously described (Bamias et al., 2008). Briefly, 10⁶ cells were incubated with mouse anti-human mAbs against CD3, VEGFR-1, VEGFR-2, VEGFR-3 or IgG1 antibodies used as isotype controls (BD Pharmingen, San Jose, CA, USA) at 4 °C for 30 min. Cells were washed twice in washing buffer (PBS containing 0.5% fetal bovine serum and 0.01% sodium azide), fixed with CellFix solution (BD Pharmingen) and subsequent analysis was performed on a three-colour fluorescence FACSCalibur cytometer using CellQuest software (Becton-Dickinson, San Jose, CA, USA). At least 10 000 gated events per condition were analysed. Specific subpopulations of T cells were identified using anti-CD4, CD8, CD56 and CD25 mAbs, as previously described (Bamias et al., 2007, 2008).

**Enzyme-linked immunosorbent assay (ELISA) determination of human VEGF**

Soluble VEGF levels in the initial ascitic fluid and in culture supernatants were determined by standard ELISA methods, using a VEGF detection kit in accordance to the manufacturer's instructions (R&D Systems).

**Immunocytochemistry**

Immunocytochemistry experiments were performed as described (Ziogas et al., 2012). Briefly, cytological specimens were prepared in a cytocentrifuge (cytospin 4, ThermoShandon, Waltham, MA, USA) and placed in ethanol for 30 min. Immunocytochemical studies were performed using the automated Ventana immunostainer (Ventana NexES; Ventana Medical Systems Inc., Tuscon, AZ, USA), using the iVIEW DAB detection kit (Ventana). Samples were incubated with either Flt-1/VEGFR-1 rabbit polyclonal Ab (Neomarkers, Lab Vision Corporation, Waltham, MA, USA) (dilution 1:25) or Flk-1/VEGFR-2 mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution 1:25), anti-CD3 mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls. The specific antibody was localised by a universal anti-mAb (DakoCytomation, Glostrup, Denmark), anti-calretinin mAb (Invitrogen, Carlsbad, CA, USA), plus relevant positive and negative controls.

**Western blotting**

Monocyte-depleted ascitic lymphocytes were grown with or without VEGF, cell lysates were prepared using 10 × 10⁶ cells per
preparation and subjected to western blotting as described before (Sambrook et al, 1989; Ziogas et al, 2012). Briefly, 50 μg of total protein from each preparation was mixed with SDS–PAGE reducing buffer (Sigma Aldrich Ltd, Dorset, UK), electrophoresed, separated proteins were subsequently transferred onto Trans-Blot Transfer Membrane (Bio-Rad Laboratories Ltd, Hercules, CA, USA). Membranes were blocked by adding 1 μg ml \(^{-1}\) anti-FcR Ab (R&D Systems) to prevent non-specific binding of the primary Ab, and further incubated with 1 μg ml \(^{-1}\) pure mouse anti-human VEGFR-2 Ab (R&D Systems) overnight at 4 °C. Membranes were washed for 30 min with Tris Buffered Saline-Tween 1% and subsequently incubated with 1 μg ml \(^{-1}\) of HRP-conjugated anti-mouse Ab (Cell Signalling, Danvers, MA, USA) for 1 h at room temperature. Membranes were finally washed with Tris Buffered Saline-Tween 1% (4 × 15-min washes) and underwent development using ECL analysis system (Amersham Pharmacia Bicosciences, Piscataway, NJ, USA), according to the manufacturer’s protocol with final exposure to preflashed X-ray film.

Cytotoxicity assay

The cytotoxic activity of cultured lymphocytes was determined in a standard 4-h \(^{1}C\)-release assay against K562 (human erythroleukemia), Daudi (Burkitt’s lymphoma) cells lines and cryopreserved autologous tumour cells from each patients’ ascites. Targets (10\(^{6}\) cells) were labelled with 100 μCi sodium \(^{51}\)Cr chromate (The Radiochemical Centre, Amersham, Bucks, UK) for 1 h at 37 °C. Effector cells were coincubated with target cells at ratios of 100:1, 50:1, 25:1 and 12.5:1. After 4 h, 100 μl of each well’s supernatant was removed and isotope was counted in a γ-counter (1275 Mini-gamma LKB Wallac, Turku, Finland). To determine maximal and spontaneous isotope release, target cells were incubated with 3 N HCl and in plain medium, respectively. In order to determine maximal release, the isotope was counted in the well’s supernatant and subsequently washed with 3 N HCl and in plain medium, respectively. In all experiments, spontaneous isotope release did not exceed 10% of maximal release. Percentage of specific cytotoxicity was calculated as described (Ziogas et al, 2012).

Statistical analysis

Statistical tests were performed using the SPSS software (version 14.0) (IBM, Armonk, NY, USA). Differences in related cell populations at different time points were analysed using the paired Wilcoxon test. Non-parametric Kruskal–Wallis H and Mann–Whitney U-tests were used to compare medians of cell populations across different time points. All P-values were two-sided and 5% was chosen to denote significance.

RESULTS

Vascular endothelial growth factor suppresses T-cell proliferation

Ascites from 15 ovarian cancer patients were used in this study and were selected on the basis of low NK- and B-cell content (<3–4%). Mononuclear cells were depleted from monocytes by means of plastic adherence and expanded in cultures with anti-CD3 and IL-2 for 14 days. On day 14, >95% of cells were CD3 positive (+). There was a gradual decrease of CD4 + cells (and a corresponding increase of CD8 + cells), from a basic mean value of 58.4% (s.e. 5.2) to 37.7% (s.e. 7.2) on day 14 (P = 0.036). On day 0, in all samples the regulatory T-cell subpopulation (Tregs; CD4 + CD25\(^{hi}\)) was low (<3%), but significantly increased from a mean value of 0.95% (s.e. 0.22) to 3.2% (s.e. 0.59) (P = 0.0012) by day 14. CD3 + ID56 + cells constituted ≤6% of the initial T-cell population and did not show any consistent trend for increase by day 14. Identical changes were also observed in cell cultures exposed in vitro to VEGF, suggesting that these were the result of culture conditions and not due to the effect of this factor.

Activated T cells express VEGFR-2

To detect the expression of different types of VEGFR on anti-CD3 and IL-2-activated T cells, lymphocytes from five patients were analysed for the expression of VEGFR-1, -2 and -3 by means of flow cytometry, immunocytochemistry and western blotting. Although VEGFR-1 or -3 were not expressed (data not shown), VEGFR-2 was detected on the surface of CD3 + T cells (Figure 2A). More specifically, on day 0, <5% of the cells expressed VEGFR-2,
Vascular endothelial growth factor is secreted by activated T cells

To investigate whether activated T cells secrete VEGF, T cells from three patients were expanded in cultures with anti-CD3 and IL-2 for 14 days and VEGF levels in culture supernatants were measured by ELISA at 7 and 14 days. There was a reduction in the amount of soluble VEGF from day 7 to day 14 (mean values: 535 (s.e. 439) vs 194 (s.e. 95) pg ml\(^{-1}\), \(P = 0.467\)), indicating that activated T cells have the ability to secrete VEGF in their culture environment. It should be noted that the mean VEGF value in the ascites of the same patients was 1873 pg ml\(^{-1}\) and these high ascitic VEGF levels did not correlate with the amount of VEGF determined in T-cell culture supernatants (\(P = 0.299\) and \(P = 0.117\), for days 7 and 14, respectively).

Vascular endothelial growth factor reduces the cytotoxic activity of T cells

To assess the dose-dependent effect of VEGF on T-cell cytotoxicity, various concentrations of rVEGF (0.1–100 ng ml\(^{-1}\)) were repeatedly added to T-cell cultures from the ascites of five ovarian cancer patients over 14 days. For statistical analyses, data from all effector:target ratios at each concentration were pooled together. On the day of culture initiation, isolated lymphocytes exhibited marginal cell lysis (\(\leq 10\%\)). Low VEGF concentrations did not reduce T-cell cytotoxicity on days 7 and 14, whereas VEGF concentrations \(\geq 1\) ng ml\(^{-1}\) considerably inhibited T-cell-mediated target cell lysis at all effector to target cell ratios tested (Figure 4). Specifically, statistically significant decrease of T-cell cytotoxicity was noticed by 1–100 ng VEGF ml\(^{-1}\) against K562 and Daudi (in all cases, \(P = 0.012\)) and by 5–100 ng VEGF ml\(^{-1}\) against autologous cancer cells (\(P = 0.018\)). The reduction of target cell lysis was most prominent on day 14, indicating that both the concentration and the prolonged presence of VEGF contribute to T-cell cytotoxic activity suppression. The cytotoxicity against
autologous cancer cells was lower than against the other two targets ($P = 0.012$) for controls and VEGF-treated cells.

**DISCUSSION**

In cancer patients, immune system functions are impaired. Tumours have effectively developed several mechanisms of escaping immune surveillance. These include the production of immunosuppressive factors, comprising cytokines, such as TGF-beta and IL-10, which act directly or indirectly on T cells and/or monocytes (Zou, 2005). Solid tumours are known to also produce abundant amounts of VEGF, which besides promoting the extension of tumour vasculature, has been shown to hinder dendritic cell functional maturation, a key element for tumour antigen-specific activation of T cells (Gabrilovich et al, 1996). Our group has previously shown that in patients with advanced ovarian cancer, VEGF levels in ascites are inversely correlated with activation protocols used by Basu et al (2010) and herein (24 h with 1 $\mu$g ml$^{-1}$ anti-CD3 vs 7–14 days with 25 ng ml$^{-1}$ anti-CD3 and IL-2, respectively). Most importantly, as Basu et al (2010) studied VEGFR expression on antigen-experienced memory (CD4$^+$/CD45RO$^+$) T cells, a possibility to be considered is a differential expression pattern of VEGFRs among selected T-cell subsets, correlated with subsequent cell activation or suppression via pleiotropically acting cytokines such as VEGF.

Vascular endothelial growth factor exhibited a functional inhibitory effect on T lymphocytes. This was evident against K562, Daudi and autologous cell targets. The minimal VEGF concentration (1–5 ng ml$^{-1}$), which suppressed T-cell proliferation and function, is in line with the levels of VEGF measured in the ascites of ovarian cancer patients (Bamias et al, 2008). The clinical relevance of our findings is further enhanced by the fact that, as already reported by our team, VEGF concentration > 1.9 ng ml$^{-1}$ in ascites was associated with adverse prognosis (Bamias et al, 2008).

The capacity of T cells to lyse autologous cancer cells was low compared with that against K562 and Daudi and was further reduced by VEGF. Several reasons can justify this observation. First, in our culture system, the presence of anti-CD3 in conjunction with IL-2 in the absence of antigenic stimulus, favours the non-specific activation of all cells bearing T-cell receptors. Consequently, autologous tumour antigen-specific T-cell clones are not preferentially expanded and may finally comprise a minor subpopulation among proliferating T cells (Crossland et al, 1991). Second, the suppressive milieu of the ascites is likely to hinder the functionalities particularly of tumour antigen-specific T cells, resulting in their poor propagation ex vivo (Ioannides et al, 1991). Finally, autologous tumour cells are often reported to escape T-cell-mediated killing by down-regulating MHC molecule and/or tumour antigen expression (Igney and Krammer, 2002). Therefore, the fact that we observed killing of autologous cells is significant by itself. This, viewed in the context of our other findings, supports the hypothesis that T cells residing in an environment rich in tumour cells, are initially activated by tumour-associated antigens, but this activation leads to VEGF expression and finally to suppression, induced by the increased VEGF levels present within ascitic fluids. It is also of importance to stress here, that the action of T cells against autologous cancer cells is a major feature of this study compared with our previous paper (Ziogas et al, 2012).

We and others have previously shown that secretion of VEGF by peripheral blood T cells can be induced by IL-2 and anti-CD3 (Zou, 2005; Mikko et al, 2009; Shin et al, 2009; Ziogas et al, 2012). In this paper, we further demonstrate for the first time that anti-CD3 and IL-2, apart from their mitogenic effect, induce VEGF secretion from autologous-derived T cells as well. This phenomenon may have implications for the physiology and pathophysiology of the immunological anti-tumour response, tumour angiogenesis and, eventually, tumour escape. It is generally accepted that an effective anti-tumour response includes primarily the recognition of tumour antigens by T cells, the subsequent activation of T cells through the T-cell receptor (this process can be mimicked by stimulating T cells with anti-CD3), and the release of immunosuppressory factors from immune cells, such as IL-2, which sustains T-cell proliferation. As this study shows, such immunological processes induce the synthesis of VEGF. This event may suggest that T-cell-derived VEGF could promote tumour angiogenesis as a result of a paracrine interaction with the tumour stroma. Activated T cells induce the expression of VEGFR-2 on their surface, thus
In summary, we have demonstrated for the first time that T lymphocytes derived from ovarian cancer patients’ ascites secrete VEGF and express VEGFR-2 on their surface in response to their activation. This in turn, promotes the suppression of activated T cells by the VEGF produced by the tumour, thus suggesting an immunosuppressive effect. These findings may have clinical relevance, particularly after recent results suggesting a benefit by the addition of the anti-VEGF monoclonal antibody, bevacizumab, to chemotherapy for advanced ovarian cancer (Burger and Brady, 2010; Markman, 2011). The rationale is that anti-VEGF and/or VEGFR-2 therapies may target not only angiogenesis but also tumor escape from immune surveillance. If this is true, the degree of the effect of VEGF on the immune system may be used to select patients likely to benefit from such approaches.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)
Dimopoulos MA, Bamias A (2012) VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. *Int J Cancer* 130: 857–864

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