Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03212.x

Interleukin-6 and vascular endothelial growth factor release by renal cell carcinoma cells impedes lymphocyte–dendritic cell cross-talk

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Accepted for publication 15 August 2006
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Summary

Anti-tumour T cell response requires antigen presentation via efficient immunological synapse between antigen presenting cells, e.g. dendritic cells (DC), and specific T cells in an adapted Th1 cytokine context. Nine renal cell carcinoma (RCC) primary culture cells were used as sources of tumour antigens which were loaded on DC (DC-Tu) for autologous T cell activation assays. Cytotoxic activity of lymphocytes stimulated with DC-Tu was evaluated against autologous tumour cells. Assays were performed with 75 grays irradiated tumour cells (Tu irr) and with hydrogen peroxide ± heat shock (Tu H_2O_2 ± HS) treated cells. DC-Tu irr failed to enhance cytotoxic activity of autologous lymphocytes in seven of 13 assays. In all these defective assays, irradiated tumour cells displayed high interleukin (IL)-6 and vascular endothelial growth factor (VEGF) release. Conversely, when tumour cells released low IL-6 levels ($n = 4$), DC-Tu irr efficiently enhanced CTL activity. When assays were performed with the same RCC cells treated with H_2O_2 + HS, DC-Tu stimulation resulted in improved CTL activity. H_2O_2 + HS treatment induced post-apoptotic cell necrosis of tumour cells, totally abrogated their cytokine release [IL-6, VEGF, transforming growth factor (TGF)-β1] and induced HSP70 expression. Taken together, data show that reduction in IL-6 and VEGF release in the environment of the tumour concomitantly to tumour cell HSP expression favours induction of a stronger anti-tumour CTL response.

Keywords: cytokine, dendritic cell, heat shock protein, lymphocyte, renal cell carcinoma

Introduction

The capacity of the immune system to reject tumours is supported by both innate immune cells such as natural killer (NK), NK T, γδ T cells and by cytotoxic T lymphocytes (CTL) [1–4]. The activation of specific CTL able to kill tumour cells [5,6] involves a cross-talk between dendritic cells (DC) [7] which present tumour antigenic epitopes and naive T cells. The reproduction of T cell priming *in vitro* requires knowledge of the capture of antigens by DC [8] and the cytokinic environment at the time of priming, which can skew the immune response toward T helper 1 (Th1), Th2 response or tolerance [9]. The use of tumour biopsy material as source of antigens is particularly attractive for clinical applications as it contains the patient's own specific array of tumour-associated antigens. Interest in whole tumour cells is enhanced by studies demonstrating that tumour cell lysates [10], apoptotic [11] or necrotic tumour cells [12,13] can induce specific stimulation of class I-restricted CTL. However, the putative release of immunosuppressive cytokines by tumour cells during DC loading could impede T cell activation and may be a limit for the use of whole tumour cells in immunotherapy protocols [11]. Thus, the choice of treatment to induce tumour cell death is a crucial point, as it could influence cytokine release by tumour cells.

Renal cell carcinoma (RCC) cells are known to secrete both interleukin (IL)-6 and vascular endothelial growth factor (VEGF) [14,15]. These molecules present mitogenic and immunosuppressive effects [16–18]. IL-10 and transforming growth factor (TGF)-β1 also contribute to immunosuppression in cancer [18–20]. In this study, we investigated IL-6, VEGF, IL-10 and TGF-β1 production by RCC either spontaneously and after killing treatments and

evaluated the cytotoxic activity of tumour-specific CTL stimulated with DC loaded with tumour cells.

Materials and methods

Patients and biopsies

Nine RCC patients, 48 ± 20 years of age, without previous therapy, underwent curative nephrectomy. Tumours were classified according to tumour size–lymph nodes–metastases (TNM) staging (five T3, two T2, two T1) and evaluated with the Fuhrman grading system (six grade III and three grade IV) [21]. Two patients were metastatic at time of surgery. Tumour biopsies ($n = 9$), proximal lymph nodes ($n = 4$) and peripheral blood ($n = 7$) samples were collected.

Cell culture

DC were generated within 7 days from monocytes in X-Vivo 10 medium with 10% fetal calf serum (FCS, Invitrogen, Cergy Pontoise, France), 1000 IU/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) (Schering-Plough, Huningue, France) and 400 IU/ml IL-4 (Promocell).

Lymphocytes isolated either from blood [peripheral blood lymphocytes (PBL)], tumour [tissue-infiltrating lymphocytes (TIL)] or proximal lymph nodes (LNL) were cultured for 15–27 days in X-Vivo 15 medium with 10% FCS, 2% L-glutamine, 2% pyruvate, 1% non-essential amino acids and 150 IU/ml IL-2 (Chiron, Heidelberg, Germany). Tumour biopsies were minced into small pieces and cultured for a short time (< four passages) in RPMI-1640 medium (Eurobio, Suresnes, France) containing 10% FCS. Tumour cells were then recovered and treated with either 75 grays irradiation (Tu irr) [22] or with 1 mM hydrogen peroxide (H_2O_2, Sigma, Saint Quentin Fallavier, France) for 12 h (Tu H_2O_2) [13]. H_2O_2 treatment could be combined with heat shock for 30 min at 44°C (Tu H_2O_2 + HS).

Treated tumour cells were incubated, without previous washing, overnight, with DC in a 1 : 10 (DC : tumour cell) ratio. DC were then co-cultured with lymphocytes in a 1 : 100 (DC : lymphocyte) ratio during 7 days. Controls were performed with non-stimulated lymphocytes evaluated at the same time.

Flow cytometry analysis

Cells were stained by monoclonal antibodies (mAb) against CD3, CD4, CD8, CD56 (Beckman-Coulter, Marseille, France) and HSP70 (Stressgen, Vancouver, Canada). Apoptotic cells were detected by annexin V-propidium iodide assay (Immunotech). Data were acquired on a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, USA).

Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

RCC cells ($5{\cdot}10^4$) were incubated for 48 h in 3 ml of medium. IL-6, IL-10, VEGF and TGF-β1 concentrations were measured in supernatants with ELISA kits purchased from Biosource, Montrouge, France; R&D Systems, Lille, France; Becton Dickinson and AbCys, Paris, France, respectively. For TGF-β1 measurements, the latent form was cleaved into the active form by acid treatment [18].

Immunohistochemical cytokine detection

A representative slice of the tumour with the highest nuclear grade was selected for immunostaining. Slices were incubated at room temperature for 1 h with the primary mAb: anti-IL-6 (titre 1/40; R&D Systems), anti-VEGF (titre 1/100; Santa Cruz Biotechnology Inc., Santa Cruz, USA) or anti-IL-10 (titre 1/100; R&D Systems). Primary mAb were revealed using a biotin–streptavidin detection system (Dako, Glostrup, Denmark). The cytokine labelling index (LI) was expressed as percentage of positive cells by counting at least 1000 tumour cells within at least 10 consecutive high-power fields.

Western immunoblotting

Cells were lysed and equal quantities of protein (50 μg) were separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membrane. Blots were incubated with anti-HSP70 mAb (Stressgen) and with control anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mAb (Biochain, Hayward, CA, USA). The membrane was incubated with peroxidase-labelled anti-mouse IgG (Bio-Rad, Jury-sur-Seine, France) and bands were revealed using the enhanced chemiluminescence kit from Amersham, Aylesbury, UK.

Cytotoxicity assays

RCC cells ($5{\cdot}10^3$) labelled with ^{51}Cr sodium chromate (1 mCi/10^6 cells, Amersham) were co-cultured in RPMI-1640 medium in 96-well U-bottomed plates for 4 h with autologous lymphocytes at a 50 : 1 effector : target cell ratio. ^{51}Cr release was assessed in culture supernatants using a Top-count gamma counter (Packard Instruments, Rungis, France). Specific activity was calculated using the formula: [mean experimental counts per minute (cpm)–mean spontaneous cpm]/(mean maximum cpm–mean spontaneous cpm) × 100.

Results

Cytokine release by RCC cells

Nine RCC cultures were investigated for IL-6, VEGF, TGF-β1 and IL-10 release in culture supernatant. All but one consti-

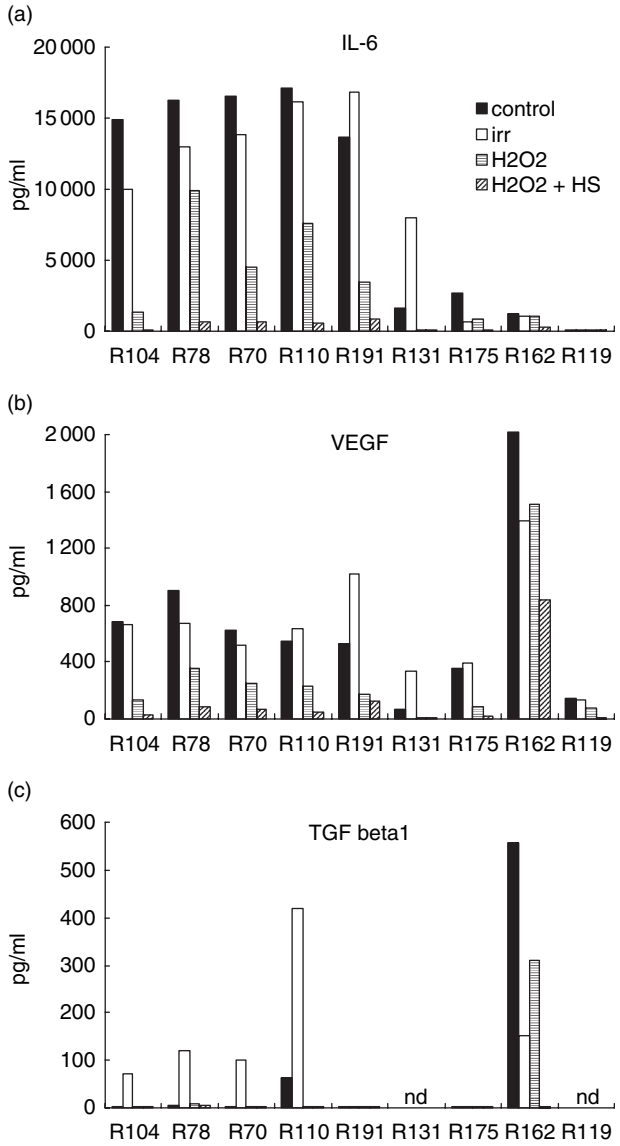

Fig. 1. Interleukin (IL)-6, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 production in renal cell carcinoma (RCC) cells. RCC cells ($n = 9$) were either untreated, irradiated (irr) or treated by hydrogen peroxide (H_2O_2) combined or not with heat shock (H_2O_2 + HS). Cells ($5{\cdot}10^4$) were incubated for 48 h in 3 ml of RPMI-1640 medium. IL-6, VEGF and TGF-β1 release was measured in supernatant of cell culture by enzyme-linked immunosorbent assay; nd = not determined.

tutively produced IL-6 at various levels ranging from 1·3 to 16 ng/ml (Fig. 1a) and could be sorted into two groups: five of nine with high IL-6 secretion (> 10 ng/ml) and four of nine with low release. VEGF was detected in all cultures, ranging from 50 to 900 pg/ml (Fig. 1b). Tumour cell irradiation, treatment performed commonly for DC loading, did not significantly modify IL-6 and VEGF secretions except for R131 and R191, which present an increased release. Interestingly, cultures characterized by high IL-6 release also present the strongest VEGF secretion except for R162. Constitutive secretion of TGF-β1 was low or undetectable except, again, for R162 (Fig. 1c). However, irradiation induced TGF-β1 release (= 100 pg/ml) for four of seven assays. Finally, IL-10 release could never be detected (data not shown). The role of contaminating endothelial cells or leucocytes in the immunosuppressive cytokine secretion was excluded with regard to the absence of $CD45^+$ or $CD31^+$ cells in flow cytometry analyses (data not shown).

In parallel, biopsy specimens of the same nine RCC were submitted to immunochemistry analysis. IL-6, VEGF and IL-10 immunostaining was variable depending on the tumour. Labelling index was from 5 to 40% (mean 14%) for IL-6, from 0 to 100% (mean 44%) for VEGF and from 10 to 100% (mean 45%) for IL-10.

IL-6 and VEGF impede DC tumour cells (DC-Tu) stimulation of lymphocytes

The cytotoxic activity of lymphocytes stimulated with DC loaded with irradiated tumour cells (DC-Tu irr) was evaluated against autologous tumour cells. For seven of 13 assays, stimulation did not improve the cytotoxic activity of lymphocytes (Fig. 2). For these unsuccessful assays, irradiated tumour cells were characterized by high IL-6 and VEGF release. Conversely, when tumour cells ($n = 4$) were characterized by low IL-6 secretion (< 10 ng/ml), stimulation was efficient and resulted in an enhancement of the cytotoxic activity. R110 was an exception because stimulation by DC was efficient despite the high IL-6 secretion.

The distribution of T cells was not modified after the DC-Tu irr stimulation either for CD8 T lymphocytes (33 ± 10 *versus* $33 \pm 20\%$, $n = 10$) or for $CD3^-$ $CD56^+$ NK cells (9 ± 6 *versus* $9 \pm 3\%$, $n = 10$).

Hydrogen peroxide and heat shock treatment of tumour cells abrogates IL-6 and VEGF production and favours stimulation of lymphocytes

H_2O_2 treatment significantly reduced IL-6 and VEGF release in all cultures (Fig. 1a,b). When cells were submitted to an

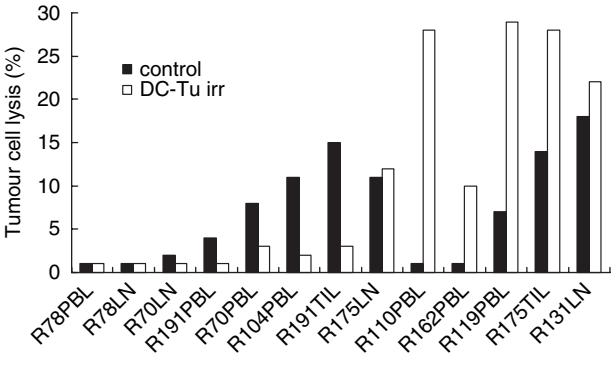

Fig. 2. Cytotoxic activity of lymphocytes after stimulation by dendritic cells (DC) loaded with irradiated tumour cells.

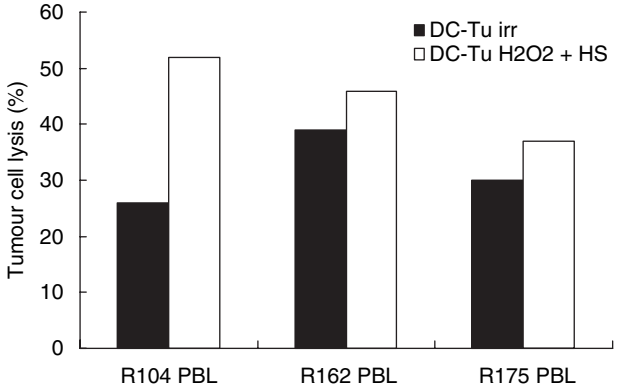

Fig. 3. Cytotoxic activity of lymphocytes after stimulation by dendritic cells (DC) loaded with hydrogen peroxide + heat shock treated tumour cells.

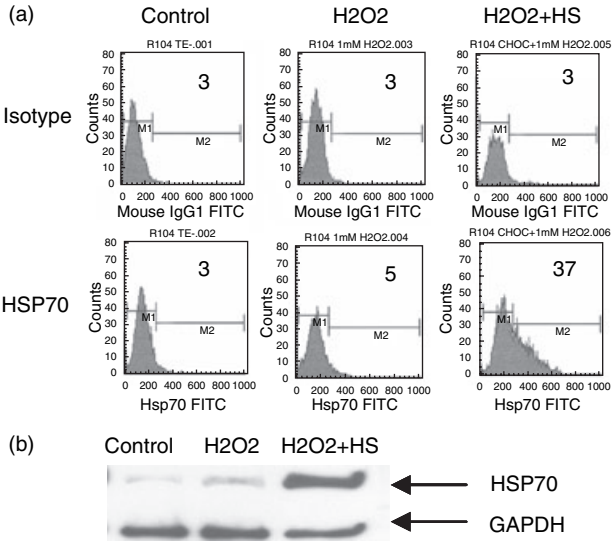

Fig. 4. HSP70 expression in renal cell carcinoma (RCC) cells after hydrogen peroxide (H_2O_2) + heat shock (HS) treatment. Tumour cells were treated during 12 h with 1 mM hydrogen peroxide (H_2O_2) or H_2O_2 plus heat shock for 30 min at 44°C (H_2O_2 + HS). Untreated cells were used as control. (a) HSP70 expression was measured by flow cytometry. Percentages of positive cells are indicated on the histograms. Representative data of seven experiments are shown. (b) HSP70 expression was measured by Western immunoblotting using an anti-HSP70 monoclonal antibody. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression was used as standard protein. Representative data of three experiments are shown.

additional heat shock (HS), IL-6 and VEGF production was completely abrogated in eight of nine assays (except R162). TGF-β1 release was also reduced after H_2O_2 or H_2O_2 + HS treatments in comparison with the constitutive (R110, R162) or irradiation-induced (R70, R78, R104) secretions (Fig. 1c). Interestingly, when tumour cells treated with H_2O_2 + HS were used for DC pulsing, cytotoxic activity of lymphocytes was enhanced in comparison with irradiated tumour cells (Fig. 3).

Hydrogen peroxide and heat shock treatment of tumour cells induces cell necrosis and HSP70 expression

Unlike irradiation, H_2O_2 and H_2O_2 + HS treatments increase the percentage of cell necrosis in RCC cell lines in comparison to control cells (Table 1). Interestingly, HSP70 expression was evidenced only after H_2O_2 + HS combined treatment either in flow cytometry analysis (Fig. 4a) or in Western immunoblotting assays (Fig. 4b).

Discussion

Several studies have demonstrated, in patients with cancer, a blockade in the maturation process of DC related to the release of cytokines and other soluble factors [23,24]. In RCC, IL-6 and VEGF are known as the main immunosuppressive factors [17,25].

In this study, we investigated IL-6, VEGF, TGF-β1 and IL-10 release in RCC cultures and the influence of killing treatments on this secretion. A constitutive expression of large amounts of IL-6 (> 10 ng/ml) associated with VEGF secretion (> 500 pg/ml) was found in five of nine RCC cultures [26]. With regard to TGF-β1 secretion, only two of seven primary RCC cultures released some detectable amounts while high TGF-β1 production was observed in culture from one metastatic patient. This result corroborates a study indicating that TGF-β1 production is enhanced in advanced RCC tumour stage [27]. The presence of immunosuppressive cytokines during the DC loading could impede the induction of a strong immune response and this question is worth studying in the vaccination protocols.

To date, irradiation is used commonly to induce tumour cell death. However, our assays showed that viability of

Table 1. Effects of hydrogen peroxide (H_2O_2) associated or not with heat shock (HS) on necrosis of RCC cells.

	Control	Irradiation	H_2O_2	H_2O_2 + HS
% Necrotic cells	15 ± 17	15 ± 18	32 ± 12	34 ± 9
% Viable cells	68 ± 24	67 ± 24	60 ± 12	59 ± 10

Renal carcinoma cells (RCC) were gamma-radiated or treated with 1 mM H_2O_2 for 12 h combined or not with HS (44°C during 30 min). Necrotic cells were detected by annexin V–propidium iodide assay. Control was performed with untreated cells. $n = 6$ for control and irradiation and $n = 3$ for H_2O_2 and H_2O_2 + HS. Necrotic cells: A +, PI + viable cells: A-PI-.

gamma-radiated RCC cells was similar to untreated cells. This result could be related to the clinically known resistance of human RCC against radiation [28]. Moreover, irradiation did not change IL-6 and VEGF release. It induced, in half the assays, a TGF-β1 secretion which could be the consequence of the up-regulation of TGF-β1 gene expression following irradiation [29]. In seven of 13 assays, stimulation with DC loaded by irradiated tumour cells was insufficient to enhance cytotoxic activity of autologous lymphocytes. In all these defective assays, irradiated RCC cells were characterized by a high IL-6 and VEGF release. Inversely, when irradiated RCC cells presented low IL-6 release ($n = 4$), stimulation efficiently enhanced CTL activity. These results suggest a negative impact of IL-6 and VEGF on T cell activation. H_2O_2 and heat shock are alternative treatments to induce tumour cell death. Hydrogen peroxide induces apoptosis via the caspase activation or necrosis by decreasing intracellular adenosine triphosphate (ATP) [30], while heat shock is known to favour HSP70 expression, a molecule involved in DC maturation and cross-presentation [10,31–34]. Our results show that H_2O_2 plus heat shock succeeded in inducing necrosis of RCC cells. It allowed the abrogation of IL-6 and VEGF secretion as well as strong HSP70 expression and led to improved CTL activity.

In conclusion, our data obtained with human tumours collected from patients with RCC show that treatment of tumour cells by H_2O_2 and heat shock enhances their immunogenicity. The underlying mechanisms could be the abrogation of immunosuppressive cytokine release into the tumour environment as well as the induction of danger signal such as the chaperone protein HSP70. These results could be helpful in optimizing strategies aimed at inducing a strong CTL response against tumour cells and in favouring the development of adoptive cell therapy protocols.

Acknowledgements

We thank Matthieu Le Gallo* and Frederic Epstein (Inserm U601) for Western immunoblotting assays. Pascaline Charlot and Selim Zerrouki (Laboratoire de Génétique moléculaire et hormonologie, CHU de Rennes) for technical assistance. This work was supported by grants from the Comité Grand Ouest de la Ligue Contre le Cancer, the Faculté de Médecine de Rennes and the Groupement d'Intérêt Scientifique de Thérapie Cellulaire de Rennes, France.

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