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Original Contribution

Redox-signaling transmitted *in trans* to neighboring cells by melanoma-derived TNF-containing exosomes

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Abstract

Hydrogen peroxide is known to be involved in redox signaling pathways that regulate normal processes and disease progression, including cytokine signaling, oxidative stress, and cancer. In studies on immune surveillance against cancer, hydrogen peroxide was found to disrupt cytotoxic T-cell function, thus contributing to tumor escape. In this study, secretion of TNF-containing vesicles of rab9+ endosomal origin, termed exosomes, was investigated using GFP-TNF constructs. We observed a polarized intracellular trafficking and apical secretion of TNF-positive nanovesicles. Cell-to-cell transfer of TNF was observed in exosomes in real-time microscopy, occurring separate from the melanin/melanosome compartment. Exosomes were prepared by ultracentrifugation or immunoisolation on anti-β2-microglobulin magnetic beads. TNF as well as TNF receptors 1 and 2 were present in the exosomes as determined by Western blot, flow cytometry, and deconvolution microscopy. The functional significance of melanoma-derived exosomes was established by their signaling competence with ability to generate significantly higher ROS levels in T cells compared with sham exosomes ($P=0.0006$). In conclusion, we report here, for the first time, that TNF is found in tumor cell-derived exosomes and that these exosomes transmit redox signaling *in trans* to neighboring cells. The results are of importance for a better understanding of tumor escape mechanisms.

Keywords: TNF; Exosomes; Melanoma; Hydrogen peroxide; Redox signaling; Tumor escape mechanisms

Introduction

Hydrogen peroxide (H_2O_2) is generated in phagocytes as a host defense to inflict damage to engulfed microorganisms, but H_2O_2 also supports proliferation, differentiation, and migration and affects numerous intracellular signaling pathways including growth factors and cytokines such as platelet-derived growth factor, epidermal-derived growth factor, insulin, TNF, and interleukin-1 (IL-1) [1]. The role of cytokines, particularly TNF in malignant melanoma, has been studied extensively. While most studies address the production of TNF by immune cells that infiltrate the tumor tissue, melanoma cells have been shown to express TNF both in vitro and in vivo [2]. Intracellular expression of TNF in melanoma cells confers resistance against the TNF-mediated killing launched by invading immune cells, partly due to the down-regulation of TNF receptors (TNFRs) on the melanoma cells [3]. In vivo, the development of tumor-related anergy could be linked to the appearance of hydrogen peroxide induced oxidative stress that blocks effector T-cell function [4]. In addition, tumor-derived exosomes that inhibit T-cell functions have been isolated from patients' sera [5].

Exosomes are nanovesicles of 40 to 90 nm in size that are produced by various cells in the body under normal physiological conditions such as immune suppression during pregnancy [6–8] as well as during disease conditions, e.g., inflammation

Abbreviations: β_2m, β_2-microglobulin; carboxy-H_2DCFDA, (5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; FCS, fetal calf serum; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; Ig, immunoglobulin; IP, immunoprecipitation; IL, interleukin; MFI, mean fluorescence intensity; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PFA, paraformaldehyde; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TNF, tumor necrosis factor; TACE, TNF-α-converting enzyme/ADAM17; TNFR, tumor necrosis factor receptor; Trx, thioredoxin; TrxR, thioredoxin reductase; WB, Western blot.

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and cancer [9]. Exosomes are also explored in dendritic cell-based tumor vaccines [10].

The expression of TNF in primary human melanomas in situ has been correlated to the decreased infiltration of CD3-positive cells [11]. Trying to explore the biological role of TNF in melanomas, we previously studied the expression of various cytokines and redox-active proteins such as thioredoxin 1 (Trx1) and Trx reductase 1 (TrxR1) in a panel of cancer cell lines [12–14], and found that despite a high intracellular expression, soluble TNF was not released into the supernatants [15] in contrast to macrophages that secreted high levels of TNF as well as redox-active proteins.

In the present study, we wanted to explore (i) if TNF was correctly processed in malignant melanoma cells; (ii) if and how it was exported from melanoma cells; and (iii) a possible mechanism of immune regulation. We found that in the melanoma cell lines studied, the TNF-α-converting enzyme (TACE)/*a d*isintegrin *a*nd *m*etalloprotease domain 17 protein (ADAM17) was indeed present and TNF was correctly cleaved. However, melanoma-produced TNF was not released directly into the supernatant, but was concealed in exosome vesicles. The presence of both TNFR1 and TNFR2 was also revealed in these exosomes, confirming a previous study that found TNFR1 in exosomes released from human vascular endothelial cells [16]. Furthermore, we found that melanoma-released exosomes induced high levels of reactive oxygen species (ROS) in lymphocytes, suggesting a possible role of "immune counterattack" for tumor-derived exosomes.

Materials and methods

Cell cultures

FM3 and $FM55_{M2}$ malignant melanoma cell lines [17], THP-1 monocytic cell line (ATCC: TIB-202), and Jurkat T-cell line (clone E6-1, ATCC: TIB-152) were maintained in an RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). For stimulation, cells were treated with 50 nM phorbol 12-myristate 13-acetate (PMA) for the indicated time. For microscopic studies, if not otherwise indicated, cells were seeded at a cell density of 0.5×10^5 cells/ml, onto round coverslips of 19 mm diameter, and placed in 12-well culture plates.

Molecular biology reagents and materials

Restriction endonucleases, oligonucleotides, enzymes, and cell culture reagents were purchased from Invitrogen (Paisley, UK), unless otherwise indicated. Deep Vent DNA polymerase was purchased from New England Biolabs (Ipswich, MA), and the vectors, pEGFP-N3 from Clontech (Mountain View, CA). The following mouse monoclonal antibodies (mAbs) were used in this study: anti-human TNF clone CY-014, purchased from Innogenetics (presently distributed by Triolabs AB, Gothenburg, Sweden); biotinylated anti-human TNF (clone BAF210, R&D Systems, Minneapolis, MN); anti-TACE, clone M222, was donated by Dr. R. Black (Immunex Corp., Seattle, WA); anti-FLAG (clone M2, Sigma-Aldrich Co, St. Louis, MO); anti-TNFR1, clone htr-9 and anti-TNFR2, clone utr 1 (BMA Biomedicals, Augst, Switzerland); anti-melanosome, clone HMB-45 (DAKO, Glostrup, Denmark); anti-rab9, late endosomal marker (Abcam Ltd, Cambridge, UK); anti-golgin-97, Golgi apparatus marker, clone CDF4, A21270 (Molecular Probes/Invitrogen). As negative controls, mouse IgG1 and IgG2a Abs, as well as normal rabbit Ig (DAKO), were used. As secondary reagents, anti-mouse and anti-rabbit Ig conjugated with Alexa594 (Molecular Probes), FITC-conjugated anti-mouse Ig, biotinylated goat anti-mouse IgG, and FITC-conjugated streptavidine (DAKO) or streptavidine-Alexa488 (Molecular Probes) were used. For exosome isolation, rabbit anti-β_2-microglobulin (anti-β_2m) (DAKO, A0072) was used.

Plasmid constructs and transfection

Fig. 1B shows a schematic drawing of the plasmid constructs. (1) pEGFP-TNF fusion protein. A cDNA fragment containing the entire coding sequence of human TNF was generated by PCR using the plasmid pATHTNF (Accession Number LMBP2529, BCCM/LMBP, http://www.belspo.be/bccm/lmbp.htm) as a template with the following primers: sense 5′-TGGAATTCACACCATGAGCACTGAAAGC-3′ (EcoRI site underlined) and antisense 5′-GTCGGGTACC-CAGGGCAATGATCCCAAAG-3′ (KpnI site underlined). The resulting 722-bp product was digested with EcoRI and KpnI and subsequently subcloned in-frame into the pEGFP-N3 vector, digested in the same manner. (2) pEGFP-TNF-FLAG was generated by introducing a $(FLAG)_2$ epitope at the N-terminal of the TNF sequence. The two following complementary oligonucleotides, encoding the sequence of the $(FLAG)_2$ epitope, and containing restriction site overhangs for subcloning were used: sense sequence, 5′TCGACCATGGGAGACTA-CAAGGACGACGACGACAAGGAC TACAAGGACGAC-GACGACAAG-3′ (XhoI overhang underlined); antisense sequence, 5′-AATTCTTGTCGTCGTCCTTGTAGTCC-TTGTCGTCGTCCTTGTAG TCTCCCATGG-3′ (EcoRI overhang underlined). After annealing, the double-stranded oligonucleotide was inserted in-frame into the pEGFP-TNF plasmid digested with EcoRI and XhoI. For transient transfection of melanoma cell lines, Lipofectamine (Invitrogen) was used according to the manufacturer's instructions. Cells were transfected with 1 μg DNA for 5 h and assayed 48 h posttransfection. Alternatively, stable transfectants were selected using an optimal concentration (600 μg/ml for $FM55_{M2}$) of geneticin (Sigma-Aldrich Co.).

Immunofluorescence and flow cytometry

The paraformaldehyde (PFA)-fixation/saponin permeabilization method was used for intracellular staining, as previously described [18]. Cells were stained either in suspension for analysis by flow cytometry or on coverslips for microscopy. Primary Ab was used at a 5–10 μg/ml optimal concentration and an isotype control Ab was employed in parallel, at the same