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Combined IL-8 and TGF-β blockade efficiently prevents neutrophil infiltrates into an A549-cell tumor

Kristy Kazemfar[a], Ren Chen[d], Kimberly Nicholson[a], Domenico Coppola[b], Jun-Min Zhou[c], Xianghong Chen[c], Sheng Wei[c], George Blanck[a,c,*]

[a] Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL 33612, United States
[b] Department of Oncologic Sciences, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States
[c] Immunology Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, United States
[d] Office of Clinical Research, College of Medicine, University of South Florida, Tampa, FL 33612, United States

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ABSTRACT

Neutrophil infiltrates into tumors have been reported in certain circumstances to reduce tumor growth and in other circumstances to augment tumor growth, particularly by facilitating metastasis. Neutrophil chemotaxis can be facilitated by both interleukin-8 (IL-8) and transforming growth factor-β (TGF-β). However, the combined effects of these two cytokines on neutrophil tumor infiltrates is unknown, and we considered the possibility that studying the combined effects might resolve apparent contradictions with regard to neutrophil effects on tumor development. First, we determined that a simultaneous IL-8 and TGF-β blockade is far more efficient at eliminating the neutrophil infiltrate from an A549 derived tumor than is blockade of either cytokine alone. Blockade of IL-8 alone, led to smaller tumors, consistent with the known inhibitory role of TGF-β on A549 cell proliferation. Blockade of TGF-β alone rescued the tumor growth but led to reduced metastasis volume. Surprisingly, blockade of both cytokines rescued both tumor volume and metastasis, underscoring the difficulty of understanding the effects of complete tumor cytokine elaboration profiles by isolating the effects of only one cytokine.

1. Introduction

The secretion of cytokines by tumor cells plays a role in tumor development in many ways, for example by regulating tumor cell apoptosis or affecting the tumor stroma. However, for any given cytokine or stromal interaction there can be conflicting results. For example, IL-8 reduces the tumor growth of 5637 bladder carcinoma cells in nude mice, and this correlates with the infiltration and function of neutrophils, which have been shown to directly destroy tumor cells [1]. And, the 5637 cells secrete a low level of TGF-β, at best [2]. Also, Ting and colleagues have reported that IL-8 and neutrophils reduced the tumorigencity of the ovarian cancer cell line, OVCA 420 [3], which others have reported undergoes apoptosis when exposed to TGF-β [4,5]. However, a second ovarian cell line formed tumors in nude mice and does not undergo apoptosis in response to TGF-β [5]. Furthermore, cell lines that respond to IL-8 with either a pro-tumor or pro-metastasis effect also secrete TGF-β, particularly melanoma cell lines [2,6]. Taken together, these results raise the possibility of a pro-tumor effect for TGF-β in certain circumstances. In particular, these observations have raised the possibility that the combined secretion of IL-8 and TGF-β may be necessary for the pro-tumor effect of IL-8, which in turn could explain the conflicting reports regarding the effects of neutrophils on tumor development [7–12]. Thus, we examined the effect of blocking IL-8 and TGF-β, independently and simultaneously, on neutrophil infiltration and on tumor volume and metastasis.

2. Materials and methods

2.1. In vitro neutrophil migration assay

Chemotactic migration of neutrophils was measured with a 48-well Micro Chemotaxis Chamber (NeuroProbe Inc., Cabin John, MD). Assay media (25 μl) from A549 cells was pre-incubated with either mouse anti-human IL-8, anti-TGF-β or the isotype control antibodies for 30 min before adding to the lower chamber. Fifty microliters of a suspension of neutrophils (5×10^5/ml) from mice injected with thioglycollate 18 h prior, in plain RPMI 1640 medium, was added to the upper compartment of the chemotaxis chamber. The two compartments were separated by a 5-μm pore size, polycarbonate filter (Nucleopore Corp., Pleasanton, CA). 25 μl of recombinant human IL-

* Corresponding author at: Department of Molecular Medicine, 12901 Bruce B. Downs Bd., MDC7, Tampa, Florida 33612, United States. Tel.: +813 974 9585.
E-mail address: gblanck@health.usf.edu (G. Blanck).

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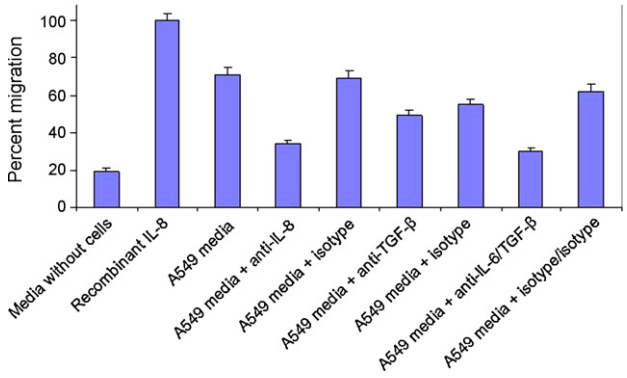

Fig. 1. Verification of the A549 derived IL-8 on mouse neutrophil migration, in vitro. Neutrophil migration was evaluated by the microwell chemotaxis chamber assay. The culture media from A549 cells were pre-incubated with either anti-IL-8, anti-TGF-β or corresponding isotype control antibodies (3 μg/ml) for 30 min before adding the media to the micro-chemotaxis chamber wells. The neutrophil migration rate was measured as described in Section 2. Results are the mean ± SE of three experiments from different preparations.

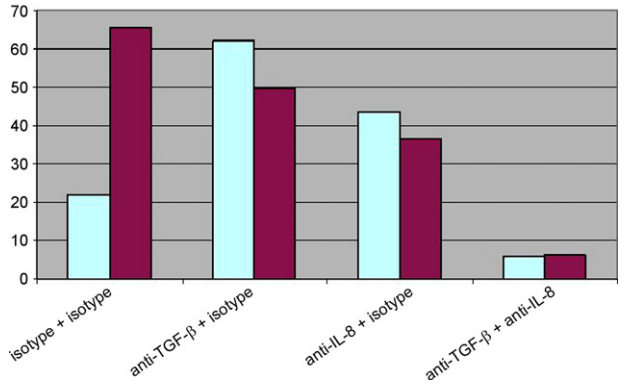

Fig. 2. Levels of mouse neutrophil infiltration into A549 tumors in the presence and absence of IL-8 and TGF-β. Bar graph of neutrophil counts in tumor mass following treatment of tumor bearing mice with different combinations of anti-IL-8, anti-TFGβ or isotype control antibodies. The dark and light shaded bars represent two separate experiments, totaling 10 mice per treatment condition. The *p*-value for isotype control only, vs. anti-IL-8/anti-TGFβ, for the light shaded experiment is 0.03; and for the dark shaded experiment is 0.0001.

8 was added to the bottom wells at a concentration of 100 ng/ml to serve as a positive control, and spontaneous migration was determined as the movement of cells toward the control medium. The chamber was incubated for 1 h at 37 °C in humidified 5% CO_2. The filter was then removed and stained with Diff-Quik (Harleco, Gibbstown, NJ). The number of neutrophils from the upper chamber that infiltrated across the filter to appear on the underside was recorded in three oil immersion fields for each well, and each experimental condition was assayed in triplicate wells.

2.2. Cell line, tumor inoculation and antibody blockade treatments

A549 lung carcinoma cells were grown in 10% fetal bovine serum in F12 media with penicillin, streptomycin, glutamate and pyruvate supplements. Mice were injected with 3 million cells subcutaneously on the flank. 100 μg of antibody was injected in a 200 μl volume of PBS in the peritoneal cavity. Thus, each mouse received a total of 400 μl of antibody solution, representing a combination of anti-IL-8, anti-TGF-β, and isotype controls, as indicated in Section 3. Anti-IL-8 and anti-TGF-β, which has neutralizing activity against all three human forms of TGF-β, and were purchased from R and D systems, cat. nos. MAB208 and MAB1835, respectively; isotype control for both anti-IL-8 and anti-TGF-β was an IgG1 purchased from R and D systems, cat. no. MAB002.

2.3. Neutrophil staining

Formalin-fixed paraffin sections of A549 tumors were cut at 3 microns, air dried overnight then heated to 60 °C to facilitate deparaffinization. Paraffin sections were deparaffinized with xylene, 2 changes, 10 min each, hydrated through descending grades of ethanol to deionized water and placed in TBS/Tween for 5 min. Following microwave antigen retrieval with 0.01 M citrate buffer, pH 6.0 (high to boiling, then 20 min on power level 5, Emerson 1100W microwave) paraffin sections were cooled for 20 min at room temperature, rinsed with deionized water then transferred to TBS-Tween. Specimens were immunostained using the Vector Elite Rabbit IgG-Peroxidase Nova Red detection kit (Vector Laboratories, Burlingame, CA) for light micrscopy. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide, non-specific binding blocked with normal goat serum and with Avidin/Biotin blocking kit reagents (Vector Laboratories). Rabbit IgG was used as the negative control reagent. The anti-Gr-1 antibody (rabbit mAb RB6-8C5) was obtained from BD Biosciences.

2.4. Tumor volume, metastasis measurements, and statistical analyses

External, flank tumor volumes were measured twice weekly using calipers for both a large and small diameter. Metastasis vol-

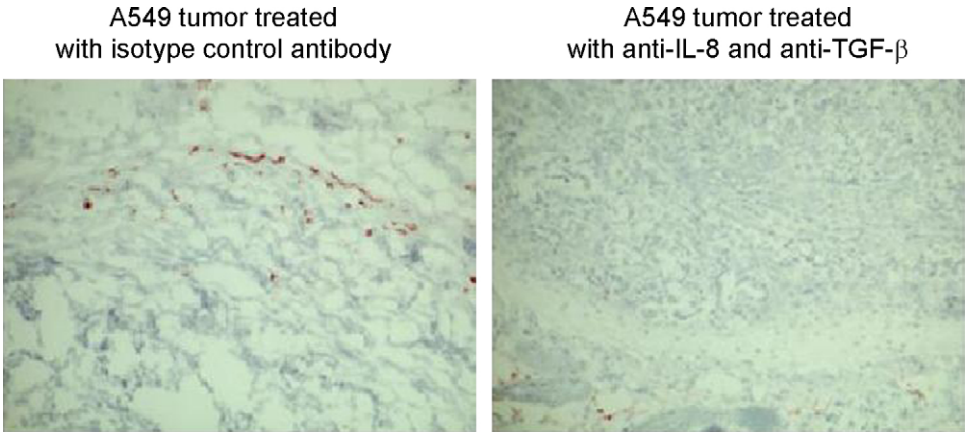

Fig. 3. Micrographs of neutrophil stains of isotype or anti-IL-8/TGF-β treated mice inoculated with A549 tumor cells. Tumor masses were stained with anti-Gr-1 antibody, as described in Section 2. Micrographs indicated tumor masses from A549 tumors treated with either isotype control or simultaneously with anti-IL-8 and anti-TGF-β neutralizing antibodies.