Decreased Levels of Both Stat1 and Stat3 in T Lymphocytes from Mice Bearing Mammary Tumors

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Abstract. Background: Lymphocytes from tumor-bearing animals have been shown to lack antitumor function. The objective of this study was to investigate the status of the signal transducers, Stat1 and Stat3, in T lymphocytes of animals bearing D1-DMBA-3 mammary tumors and to elucidate if any alterations in these signal transducers can be explained by the presence of tumor-derived factors and correlated with the lack of antitumor function in these cells. Materials and Methods: T Lymphocytes from spleens of normal and tumor-bearing mice were purified and assayed for the presence of Stat1 and Stat3 by Western blot analysis. Results: It was found that levels of both Stat1 and Stat3 were reduced in T lymphocytes of tumor-bearers not only in their active, phosphorylated form but in total protein levels. Conclusion: These findings indicate that during mammary tumor progression, alteration of various transcription factors may contribute to the down-regulation of immune function.

It has been widely reported that oncogenesis is accompanied by immunosuppression (1-4). Solid tumors secrete several factors that promote tumor cell proliferation, angiogenesis and metastasis and also produce factors that inactivate immune surveillance and promote tolerance (5-10). It is well established that T lymphocytes from tumor-bearers lack important antitumor functions, such as cytotoxicity against tumor cells, and proliferation in response to antigens or mitogens (11-13). These cells also lack critical surface molecules such as CD3 zeta chain and are prone to apoptosis (14, 15).

The signal transducer and activator of transcription (Stat) family of kinase-activated proteins are important signaling molecules in tumor cells (16-18). In particular, Stat1 and Stat3 have been linked to cancer progression (19-21). Stat1 is an interferon (IFN) α/β and γ responsive transcription factor (22, 23). It has been suggested that Stat1 promotes immunity and suppresses tumor growth (16, 19). Down-regulation of Stat1 is often observed in tumor cells, such as transformed intestinal epithelia, and Stat1−/− mice have an increased incidence of tumor formation (22, 24). In contrast, Stat3, a transcription factor responsive to interleukin (IL)-6, IL-10 and IL-23, is widely seen as a promoter of tumor growth and even considered as a potential biomarker for transformation in its active form (25-27). Many types of cancer, including breast, skin, colorectal and liver, have high levels of phosphorylated Stat3 (28-31). Inhibitors of Stat3 have largely been promoted as anticancer agents since activated Stat3 is found in many tumors and blocking Stat3 activation results in down-regulation of proinflammatory mediators such as IL-6, RANTES and IL-10, and leads to apoptosis of tumor cells (21, 32-37). Stat3 activation has been reported to stimulate tumor cell proliferation as well as to induce resistance to apoptosis (38).

Both these signal transducers also play important roles in the function of immune cells. Stat1 is activated in functional effector T lymphocytes (39). On the other hand, Stat1 is responsible for tumor-associated macrophage suppressive activity (40). Stat3 has been described as an important mediator of tumor-induced immunosuppression due to its expression in both tumor cells and immune effectors. Blockade of Stat3 in tumor cells has been shown to induce antigen-specific tumor-infiltrating T lymphocytes and dendritic cells with activated Stat3 induce T-cell tolerance that can be reversed by ablation of Stat3 activity (21). In a mouse B16 tumor model, targeted inhibition of Stat3 in hematopoietic cells induced enhanced antitumor function in dendritic cells, T-cells and NK cells leading to tumor regression (26).

Previously, we have shown that lymphocytes from mice bearing mammary tumors are functionally impaired as they are unable to proliferate, or kill tumor cells, and secrete high...
levels of tumor-promoting factors such as matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) (6, 8, 9, 41, 42). These differences would suggest an alteration in the signal transducers controlling these functions, including Stat1 and Stat3. To clarify whether mice with such tumors have altered expression of these Stats and to explore the relationship of these signal transducers in cancer immunity, a study of Stat1 and Stat3 levels in T lymphocytes was undertaken.

Materials and Methods

Mice and cell lines. Female BALB/c mice (8-12 weeks of age) used in these studies were bred in the animal facility at the University of Miami. Animal care and use was according to the guidelines of the National Institutes of Health. The D1-DMBA-3 tumor, syngeneic to BALB/c mice, is a transplantable mammary adenocarcinoma derived from a non-viral, noncarcinogen-induced neoplastic nodule after treatment with 7, 12-dimethylbenzanthracene (43). Tumors were implanted in BALB/c mice by subcutaneous injection of 1×10^6 tumor cells resulting in a measurable tumor 7-10 days post implantation. Tumors were serially transplanted from mouse to mouse and allowed to grow for different lengths of time (one to four weeks) after which, mice were sacrificed and total T lymphocytes were purified from spleens. Three to six mice were sacrificed per experiment by cervical dislocation. The T lymphocytes were purified immediately after sacrifice. All experiments were repeated a minimum of three times.

Purification of splenic T lymphocytes. Spleens were compressed in Teflon tissue homogenizers and the resulting single cell suspension was pelleted at 300g, subjected to red blood cell lysis with ACK buffer (Invitrogen, Carlsbad, CA, USA), washed and cells were counted. T lymphocytes were purified by positive selection with rat antimouse Thy1.2 using the MACS magnetic separation system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s instructions. Briefly, single-cell suspensions in cold PEB buffer (phosphate-buffered saline supplemented 2 mM EDTA and 0.5% fetal calf serum) were prepared as described above were incubated for 18 hours in the presence or absence of 5 μg/ml recombinant vascular endothelial growth factor (VEGF) obtained from R&D Systems, Minneapolis, MN, USA. Serum-free supernatants were collected after 20 hours in culture. Purified T lymphocytes and DA-3 tumor cells were used since DA-3 is a cell line derived from D1-DMBA-3. Absorbance at 450 nm was read on a Tecan SLT Rainbow Reader (San Jose, CA, USA). Absorbance values of samples were converted to nanograms per milliliter against a standard curve produced with recombinant mouse pro-MMP-9 from the ELISA kit.

Enzyme linked immunosorbent assay (ELISA). ELISA for total mouse pro-MMP-9 were carried out according to the manufacturer’s instructions (R & D Systems, Minneapolis, MN, USA). Serum-free supernatants were collected after 20 hours in culture. Labeled substrate LLVY-AMC was added to lysates and cleavage of the substrate was measured using the Wallac Victor 2 (Perkin Elmer, Waltham MA, USA) fluorometer with a 380/460 nm filter set. Cleavage was monitored every 20 minutes and a curve of relative fluorescence was generated.

T lymphocyte cultures treated with VEGF and MMP-9. T lymphocytes prepared as described above were incubated for 18 hours in the presence or absence of 5 μg/ml recombinant vascular endothelial growth factor (VEGF) obtained from R&D Systems, Minneapolis, MN. After 18 hours, the cells were pelleted and washed in complete media. Lysates of purified T lymphocytes were prepared following the manufacturer’s instructions. Labeled substrate LLVY-AMC was added to lysates and cleavage of the substrate was measured using the Wallac Victor 2 (Perkin Elmer, Waltham MA, USA) fluorometer with a 380/460 nm filter set. Cleavage was monitored every 20 minutes and a curve of relative fluorescence was generated.

Western blot analysis. Equal numbers of purified T lymphocytes were lysed using standard RIPA buffer (Santa Cruz Biotecnology, Santa Cruz, CA, USA) plus one complete mini protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) per 5 ml RIPA. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated on 10% SDS polyacrylamide gels under reducing conditions and then transferred onto Protran nitrocellulose membranes (0.45 μm pore size; Schleicher & Schuell Inc., Keene, NH) using a Trans-Blot electrophoretic cell (Bio-Rad, Hercules, CA, USA). Western blots for Stat1 and Stat3 were performed as described elsewhere (17). The results were visualized by exposing blots to CL-Xposure film (Pierce). Film was then scanned, and data were subjected to densitometric analysis using Scion Image Software (NIH). Protein levels were normalized to the hybridization signals of β-actin and reported as relative intensity.

RNA extraction. Lymphocytes (10-20 ×10^6) were purified from spleens and RNA was extracted immediately after purification using TriReagent (Molecular Research Center, Cincinnati, OH, USA). RNA was reverse-transcribed into cDNA, then the cDNA was used as a template for PCR. PCR conditions for mouse Stat3 and GAPDH were as follows: 95°C for 2 minutes, followed by 25 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds. A final extension of 72°C for ten minutes was also part of the protocol. All primers were purchased from Sigma Genosys (The Woodlands, TX, USA). Primer sequences were: Stat3 forward, GACCCGCCAACAAATTAAGA; Stat3 reverse, TCGTGGTAAACTGGAACCA; GAPDH forward, CACCACACAGTGTAGCC; and GAPDH reverse, CTGCTT CACCCACCTT. Equal amounts of PCR samples were loaded onto a 1.5% TAE agarose gel (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.3) and run for 30 minutes at 100 volts.

Chymotrypsin-like activity of the proteasome was assessed using a Proteasome Activity Assay Kit (Chemicon, Temecula, CA, USA). Lysates of purified T lymphocytes were prepared following the manufacturer’s instructions. Labeled substrate LLVY-AMC was added to lysates and cleavage of the substrate was measured using the Wallac Victor 2 (Perkin Elmer, Waltham MA, USA) fluorometer with a 380/460 nm filter set. Cleavage was monitored every 20 minutes and a curve of relative fluorescence was generated.

Mouse recombinant MMP-9 (R & D Systems) was activated in vitro following the manufacturer’s protocol. Briefly, 100 μg/ml recombinant mouse MMP-9 was activated by incubation with 0.1 p-aminophenylmercuric acetate (APMA) in TCNB (50 mM Tris [pH 7.5], 10 mM CaCl_2, 150 mM NaCl, 0.05% Brij35) overnight at 37°C. The solution was then dialyzed in TCNB for 6 hours with several changes using Slide-A-Lyzer mini dialysis units (Pierce). Activated recombinant mouse MMP-9 was diluted in media for culture. As a control, an APMA solution (without recombinant mouse MMP-9) was also dialyzed and diluted in media for culture. Either dialyzed activated recombinant mouse MMP-9 or dialyzed APMA solution was added to T lymphocytes for 18 hours.
Several studies have shown the importance of individual members of the Stat family of transcription factors in the development, maturation, and activation of T lymphocytes (44-46). To determine whether Stat1 is altered in T lymphocytes during tumor progression, BALB/c mice were transplanted with the syngeneic D1-DMBA-3 breast tumor. Whole cell lysates were prepared and analyzed by Western blot. Total Stat1 (active and inactive form) was detected using a rabbit anti-mouse antibody that detects both 89 and 91 kDa forms. A rabbit anti-actin antibody was used to control for gel loading errors. As shown in Figure 1A, Stat1 protein decreased in splenic T-cells from mice with increasing tumor burden and was barely detectable by week four of tumor growth. There was a 90% decrease in relative band intensity between the normal lymphocytes and lymphocytes from a four-week tumor-bearer (Figure 1B). This is consistent with the fact that these lymphocytes are, in general, poorly functional, having impaired cytotoxic ability, low production of IFN-γ and low levels of proliferation in response to mitogens (8, 47).

Since increased active Stat3 has been linked to immunosuppression in dendritic cells, and inactivation of Stat3 in T lymphocytes leads to tumor regression, we hypothesized that T lymphocytes exposed to tumor in vivo would produce more active Stat3 (21, 26). Total Stat3 (active and inactive forms) was detected using a mouse monoclonal antibody. Surprisingly, levels of Stat3 in T-cells mirrored those of Stat1 in that they were almost undetectable by the fourth week of tumor growth (Figure 2A, B). Since the lysates contain both nuclear and cytoplasmic fractions, it cannot be argued that the Stat3 was sequestered in the nucleus of the tumor-bearers’ lymphocytes. Neither can it be possible that the observed difference was due to conversion of inactive Stat3 to the active, phosphorylated form, as the antibody detects both forms. In order to confirm this observation, an antibody specific for the phosphorylated...
form of Stat3 was also used. Similar to the previous results, no active Stat3 was detected in lymphocytes from tumor-bearers activated for 30 min with plate-bound anti-CD3 and anti-CD28 (Figure 3).

To determine whether this lack of Stat3 expression was due to a block in transcription of the Stat3 gene, RNA was extracted from T lymphocytes of normal and tumor-bearing mice and levels of Stat3 transcripts were quantified by PCR of lymphocyte cDNA. Figure 4 shows that T-cells from normal and tumor-bearing mice had similar levels of expression of the Stat3 gene. GAPDH primers were also utilized as an internal control of expression. From these results, we concluded that the lack of Stat3 protein in T-cells from tumor-bearers was not due to any type of block or malfunction in the transcription of the Stat3 gene.

Truncated forms of Stat3 have been described in various cell types (48, 49). In all cases, these proteins are shortened from the carboxy-terminal end of the gene product. Most commercial antibodies to Stat3 recognize the carboxy terminus of the protein including the antibody we used (Figure 2). The possibility exists that in lymphocytes of tumor-bearers, the predominant form of Stat3 is one of the truncated types, which would not be recognized by the antibody. To explore this possibility, we utilized an antibody specific for Stat3, which recognizes the N-terminal portion of the protein and repeated the Western blot with this reagent (Figure 5). The results of this assay are similar to those shown in Figure 2. Stat3 was almost undetectable in T-cells from tumor-bearing animals, an outcome that leaves little doubt that our results are valid, and not an artifact of experimental design. Lysates of T lymphocytes from normal and tumor-bearers were also analyzed for proteasome activity since this is a reported mechanism of Stat

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**Figure 3.** PhosphoStat3 activation in T lymphocytes of normal (C) and 4-week tumor-bearing mice. T-cells from normal or tumor-bearing mice were incubated for 30 minutes with or without plate-bound anti-CD3 and anti-CD28 (2.5 μg/ml) followed by cell lysis. Total cell lysates were separated by 10% SDS-PAGE and immunoblotted. Analysis for Stat3 protein expression was performed using a rabbit anti-mouse pStat3 polyclonal antibody. Data are representative of four independent experiments.

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**Figure 4.** PCR amplification of Stat3 in normal (C) and tumor-bearing animals. Total RNA was extracted immediately after isolation of T lymphocytes from control and 4-week tumor-bearing animals. Equal amounts (1 μg) of RNA were reversed transcribed into cDNA and PCR was performed using mouse Stat3 and GAPDH primers. MW, molecular weight markers (Fermentas). Data are representative of 3 independent experiments.

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**Figure 5.** Analysis of Stat3 production using an antibody that detects variant forms. Lysates were prepared from T-cells of normal and tumor-bearing mice, separated by 10% SDS-PAGE, and immunoblotted. Analysis for Stat3 protein expression was performed using a mouse Stat3 monoclonal antibody to C-terminus (Cell Signalling)-labeled Stat3 (C) or rabbit anti-mouse Stat3 antibody to N-terminus (Becton Dickinson)-labeled Stat3 (N). Data are representative of three independent experiments.

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**Figure 6.** Proteasome activity in T lymphocytes of normal (C) and 4-week tumor-bearing mice. Lysates were prepared from T cells of normal or tumor-bearing mice and labeled substrate LLVY-AMC was added to lysates and incubated for 1 hour. Cleavage of the substrate was measured using the Wallace Victor 2 (Perkin Elmer, Waltham MA, USA) fluorometer with a 380/460 filter set. Cleavage was monitored every 20 minutes, and a curve of relative fluorescence was generated. Sample were run in triplicate and averaged. Data are representative of four independent experiments.
degradation. T lymphocytes from mice bearing mammary tumors have higher proteasome activity (Figure 6) as determined by the level of cleavage of fluorogenic peptide.

We have previously reported production of high levels of VEGF and MMP-9 in lymphocytes from mice bearing D1-DMBA-3 tumors (Table I) (41). To determine whether the high levels of these factors affect Stat3 in T lymphocytes, cells were incubated for 18 hours with either recombinant MMP-9 or recombinant VEGF. Whole cell lysates were prepared and Stat3 was detected by Western blot. Although it has been reported that VEGF affects Stat3 levels in other cell types (50), we found no effect in T lymphocytes (Figure 7); however, incubation with MMP-9 induced approximately a 50% reduction in Stat3 protein levels in T-cells from control mice (Figure 8).

Discussion

Several studies have shown a relationship between Stat activity and oncogenesis (30). High levels of constitutively active Stat3 have been reported in many tumors and this has been linked to tumor aggressiveness and suppression of the immune response (51, 52). Stat1 and Stat3 protein levels were determined in T lymphocytes of mice bearing D1-DMBA-3 mammary tumors. It was found that both Stat1 and Stat3 protein levels were greatly decreased in T-cells from tumor-bearers. Stat3 activity is controlled at multiple cellular levels including receptor signaling, DNA methylation, transcription, translation, phosphorylation, dephosphorylation,
mRNA splicing, degradation, natural dominant negative competition, and dimerization (48-57). The observed reduction in Stat3 is not due to differential translocation of Stat3 into the nucleus since whole cell lysates were utilized in these studies, nor is it due to a decrease in transcription since RNA levels were equal to those of controls. Stat3 in T cells of tumor-bearers is transcriptionally functional but must be degraded soon after its production, which could significantly alter the role of these lymphocytes in fighting tumor growth. Indeed, T-cells from tumor-bearers exhibited higher proteasome activity, a form of protein degradation known to regulate Stat3 (58), and this may be a mechanism contributing to Stat3 reduction.

A key question remains to be elucidated: what happens in the course of tumor growth to alter Stat3 availability? Tumors have long been known to secrete factors that influence their own growth. For example, VEGF and MMP-9 are commonly produced by tumors and are considered important in tissue remodeling and angiogenesis leading to tumor development (5, 41). Tumor-derived factors do not exclusively act on tumors and their microenvironment. Many have been reported to affect the host systemically, increasing numbers and activity of immunosuppressive cell populations such as regulatory T lymphocytes (Tregs) and myeloid-derived suppressor cells (MDSC), which block the antitumor response. VEGF and MMP-9 are frequently found to be secreted by tumors and have both been described as key mediators of vascular remodeling and angiogenesis in tumor models, and, in general, their presence in the tumor microenvironment indicates aggressive tumor behavior (5, 41). D1-DMBA-3 tumor cells produce high levels of MMP-9 and VEGF (41, 59). VEGF has been shown to induce Stat3 and be induced by Stat3 in other cell types (34, 50). We found that VEGF did not alter Stat3 expression in primary murine lymphocytes and, therefore, does not appear to directly contribute to the decrease of Stat3 in lymphocytes of tumor-bearers.

MMP-9 functions as a promoter of angiogenesis by releasing VEGF from the matrix, making it more bioavailable, and in this way, promotes tumor growth (19, 41). Stat3 activity has been shown to affect MMP-9 levels in certain cell types (60, 61), but there have been no reports of an effect in primary T lymphocytes. There are no studies currently in the literature that suggest MMP-9 alters Stat3 activity or production. We found that incubation of T lymphocytes with recombinant MMP-9 (activated in vitro) reduced total levels of Stat3. Whether this decline is directly due to MMP-9 or via intermediate factors including the proteasome remains unclear.

Although investigators have convincingly shown that Stat3 activity induces immunosuppression in tumor-bearers and its inhibition in T lymphocytes results in a more vigorous immune response followed by tumor regression (21, 26), we find this may not always be the case and may vary from one tumor model to another. We report that the levels of Stat3 from T lymphocytes decreased with increasing mammary tumor burden. Stat3 has been shown to be important for T-cell migration, proliferation and prevention of apoptosis, which are important components in the immune response to a growing neoplasm. Thus, in certain tumor environments, lack of Stat3 may be one factor contributing to ineffective immunity and tumor escape.

Acknowledgements

This work was supported by NIH grant, R01 CA25583. We thank Mantley Dorsey, Jr. for excellent technical assistance.

References


