Short Communication

Expression of Protein Mediators of Type I Interferon Signaling in Human Squamous Cell Carcinoma of the Skin[1]

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Abstract

IFN-based therapy has been shown to be active in the treatment of squamous cell carcinoma (SCC) of the skin and has promise for chemoprevention and treatment of several other cancers. In an effort to better understand the molecular mechanism of this activity, we have determined the expression pattern of several of the protein mediators of type I IFN signaling by immunohistochemistry in cutaneous SCC, SCC metastases, and adjacent nonmalignant epithelium from patient biopsies. All of the proteins, signal transducer and activator of transcription (STAT) 1α/β, STAT2, p48, STAT3α, and STAT3β, are expressed at varying levels in the adjacent epidermis, as well as in other epidermal and dermal cell types. For the majority of samples tested, the expression of one or more of these proteins was reduced in SCC primary tumors compared with the adjacent nonmalignant epithelial cells, as determined by manual scoring. Quantitative densitometry of several samples revealed differences that are statistically significant. Our study provides the first direct evidence for the expression of the IFN-stimulated gene factor 3 (STAT1α/β, STAT2, and p48) and STAT3α and STAT3β mediators of IFN-α/β signaling in human skin and skin-derived SCCs. These data have led to the hypothesis that the loss of IFN sensitivity may contribute to the development and progression of skin SCC.

Introduction

IFNs regulate proliferation, differentiation, and immune function (1). Type I IFNs (IFN-α and -β) bind to cell surface receptors with two distinct subunits: (*a*) IFN-α receptor 1; and (*b*) IFN-α receptor 2. The receptors activate members of the janus kinase family of receptor-associated tyrosine kinases. These kinases then phosphorylate STATs,[3] resulting in their translocation to the nucleus, where they can modulate the transcription of several genes (2). Binding of IFN-α to IFN-α receptor 2 triggers phosphorylation of the janus kinase 1 and tyrosine kinase 2, which subsequently phosphorylate STAT1 and STAT2 on tyrosine 701 and tyrosine 690, respectively (1). The phosphorylated (activated) STAT1 and STAT2 proteins complex with p48 protein to form the ISGF3 transcription factor (3). After translocation to the nucleus, ISGF3 activates transcription by binding to consensus DNA sequences called IFN-stimulated response elements found in the promoters of most type I IFN-responsive genes (3). In addition to activating the ISGF3 pathway, type I IFNs can induce the phosphorylation and DNA binding of STAT3, a STAT family member that is required for src oncoprotein-mediated oncogenesis (4). STAT3 homodimers or STAT3/STAT1 heterodimers can activate transcription of a unique set of genes containing *sis*-inducible elements (5) and activate the phosphatidylinositol 3'-kinase pathway (6).

IFN-based therapy has been used successfully for the treatment of several malignancies (7), including SCC of the skin, with greater activity in premalignancies and locally advanced SCC than in metastatic disease (8). To better understand the molecular mechanism of IFN action in SCC, we studied the expression pattern of type I IFN signaling proteins in clinical patient samples from a skin SCC trial.

Materials and Methods

Surgical Specimens. Baseline surgical specimens of aggressive skin SCC came from a Phase III trial of IFN-based therapy (National Cancer Institute Grant CA88233). Aggressive tumors were defined as having met one or more of the following criteria: (*a*) size greater than or equal to 2 cm in diameter; (*b*) perineural invasion; (*c*) nodal involvement; or (*d*) deep structure (*e.g.,* muscle) involvement. Sixteen specimens from 12 patients who had surgery between September 1996 and May 1998 at The University of Texas M. D. Anderson Cancer Center were used in this study. These specimens were selected to include tumor and adjacent nonmalignant tissue. The specimens were processed by routine fixation in 10% neutral Bouin's fixative and embedded in paraffin. All specimens were cut to 4-μM sections and attached to lysine-coated slides.

Immunostaining of Paraffin Sections. For each antibody, whenever possible, the entire set of tissue specimens was processed and immunostained at the same time to ensure a valid comparison between samples. Sections were deparaffinized in xylene (three times, 7 min each, room temperature) and rehydrated by stepwise washes in decreasing ethanol/H_2O ratio (100% :50%), followed by soaking in water). Antigen retrieval was achieved by boiling slides under pressure for 1 min in 0.01
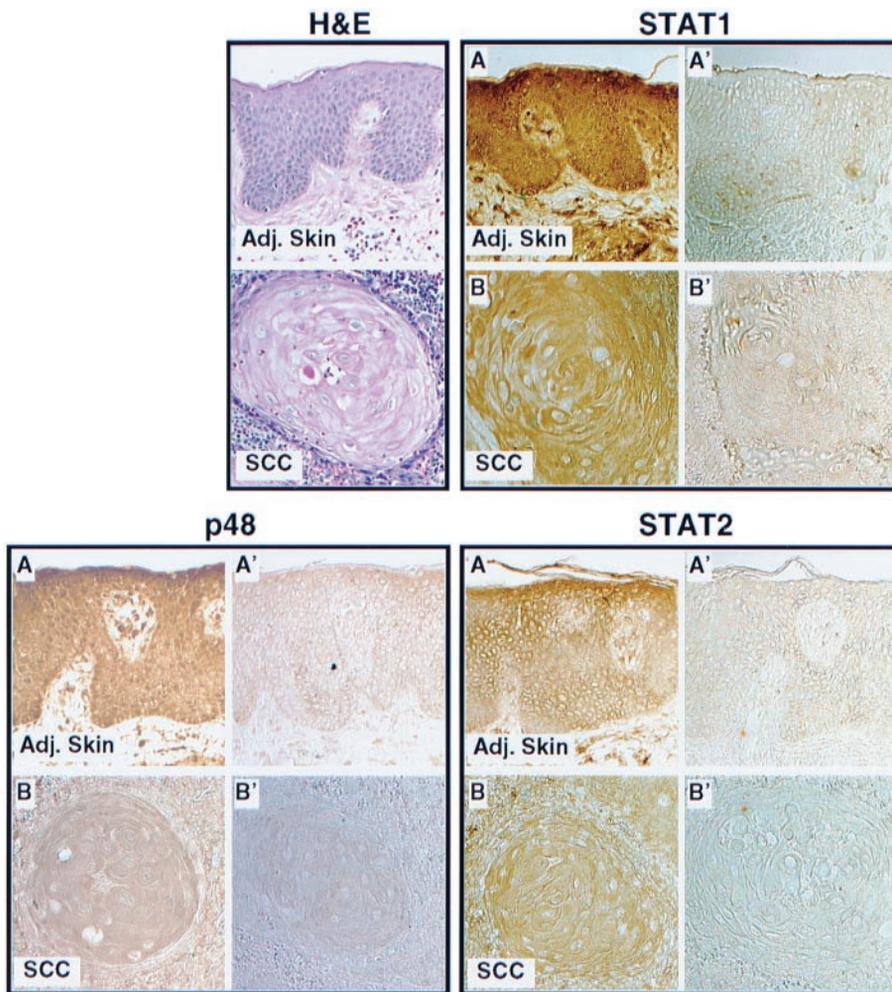

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[3] The abbreviations used are: STAT, signal transducer and activator of transcription; SCC, squamous cell carcinoma; ISGF3, IFN-stimulated gene factor 3; AS, adjacent skin.
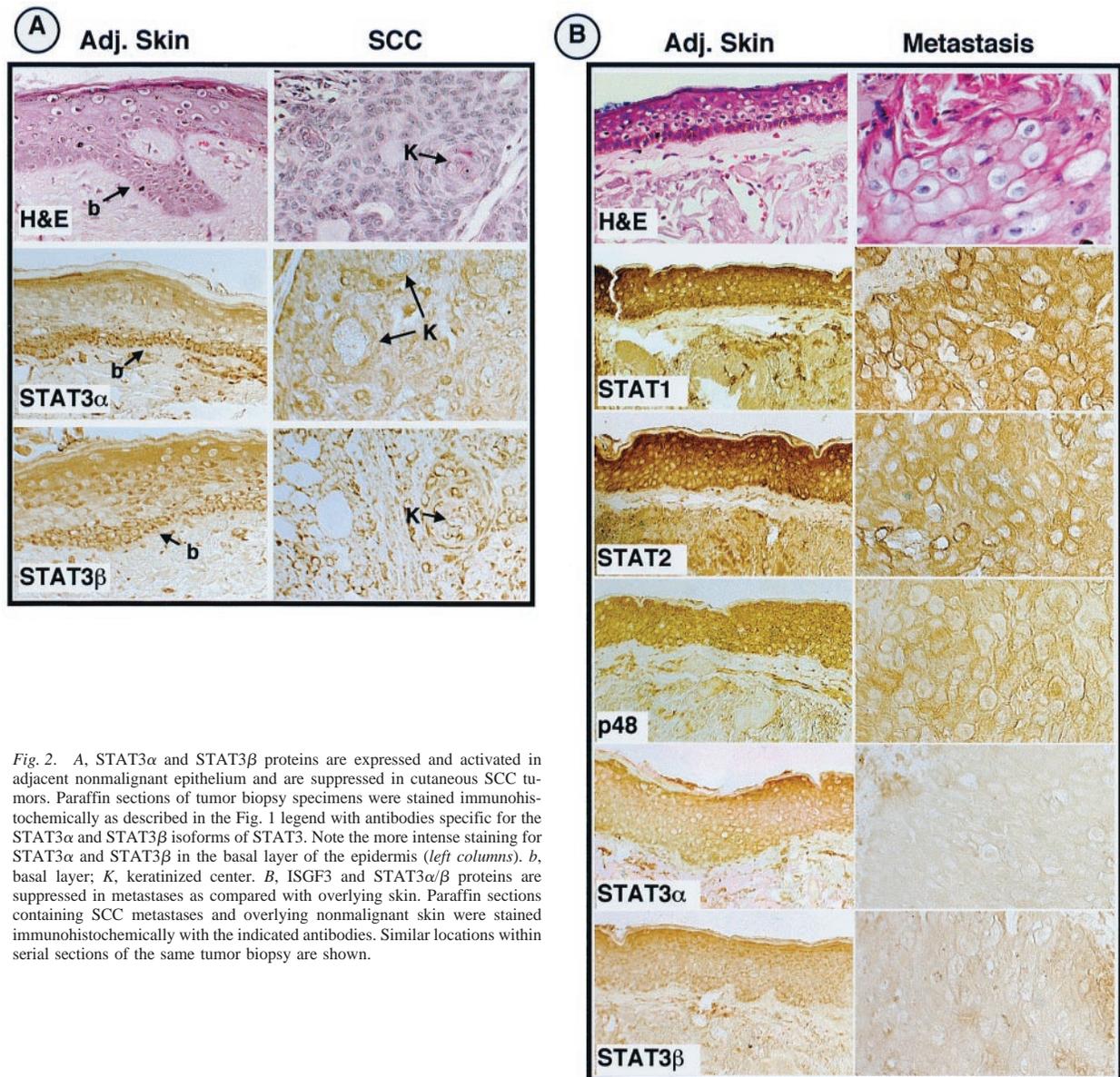
Fig. 1. The ISGF3 proteins are expressed in adjacent nonmalignant epithelium and are suppressed in cutaneous SCC tumors. *A* and *B*, thin sections (4 μM) of paraffin-embedded tumor biopsy specimens were stained immunohistochemically with antibodies for the indicated proteins. *A*, *Adj. Skin* indicates nonmalignant epithelium found adjacent to tumor tissue. *B*, *SCC* indicates SCC tumor tissue from the same slide as the normal tissue. *A'* and *B'*, sections were probed as described in *A* and *B*, except that a 10:1 (w:w) ratio of immunizing peptide:antibody was used in the primary antibody incubation step.

M sodium citrate buffer (pH 6.0), followed by cooling to room temperature. Slides were treated with 1% hydrogen peroxide for 30 min with shaking, followed by repeated washes in PBS and water. Sections were incubated with 20% normal horse serum for 1 h at room temperature to block nonspecific antigen sites. After washing three times in PBS, slides were incubated for 4 h at room temperature with appropriate dilutions of the primary antibodies. Secondary antibody detection was performed according to the instructions for the Vectastain Elite ABC kit. The STAT1α/β (SC-592 and SC-346, both specific for both the α and β splice isoforms of STAT1), STAT2 (SC-476), and p48 (SC-496) rabbit polyclonal antibodies and corresponding immunizing polypeptides were from Santa Cruz Biotechnology. An additional mouse monoclonal antibody for STAT1α/β (G16920) was obtained from Transduction Laboratories. For peptide blocking experiments, all procedures were identical to the normal staining, except that a 5:1 and 10:1 (w:w) ratio of either immunizing or irrelevant peptide:antibody was used during the primary antibody incubation step. The generation and characterization of the anti-STAT3α and anti-STAT3β isoform-specific antibodies were as described previously (9). The specificity of the antibodies was further verified by Western blotting (data not shown). Duplicate control specimens receiving the second antibody only did not stain. Slides were photographed at $\times$200 magnification under oil immersion with a Quantix charge-coupled device camera.

Densitometric Quantitation of Protein Expression. Digital images of both tumor and normal skin were captured under identical light intensity, exposure time, and camera settings with a Photometrics Quantix digital camera at a $\times$200 magnification using a blue filter. Image files were analyzed with IPLabs quantitation software. Three different fields were randomly chosen for adjacent nonmalignant skin or tumor and averaged for each specimen. Note that it was necessary to omit counterstaining of the quantitated samples to allow densitometric quantitation of only the brown color of the converted diethylaminobenzene peroxidase substrate.

Results

Skin Is a Potential Target Tissue for Type I IFNs. Sections were probed with antibodies for STAT1α/β, STAT2, p48, STAT3α, and STAT3β. To control for nonspecific binding of primary antibodies, duplicate samples were incubated with the STAT1α/β, STAT2, and p48 antibodies along with either the corresponding immunizing peptides or an irrelevant peptide. In all cases, only the immunizing peptide blocked staining (Fig. 1*A'* and *B'*; data not shown). Photographs of nonmalignant epithelium and SCC (Figs. 1 and 2*A*) or nonmalignant epithelium and metastasis (Fig. 2*B*) are taken from the same slide. H&E stains are from the same set of serial sections stained with the indicated antibodies (Figs. 1 and 2). All of the proteins

Fig. 2.   *A*, STAT3α and STAT3β proteins are expressed and activated in adjacent nonmalignant epithelium and are suppressed in cutaneous SCC tumors. Paraffin sections of tumor biopsy specimens were stained immunohistochemically as described in the Fig. 1 legend with antibodies specific for the STAT3α and STAT3β isoforms of STAT3. Note the more intense staining for STAT3α and STAT3β in the basal layer of the epidermis (*left columns*). *b*, basal layer; *K*, keratinized center. *B*, ISGF3 and STAT3α/β proteins are suppressed in metastases as compared with overlying skin. Paraffin sections containing SCC metastases and overlying nonmalignant skin were stained immunohistochemically with the indicated antibodies. Similar locations within serial sections of the same tumor biopsy are shown.

appear to be expressed in the adjacent nonmalignant epidermal tissue, indicating the potential responsiveness of skin to IFN-α (Fig. 1*A, STAT1, STAT2,* and *p48*; Fig. 2*A, left panels*). Of 16 pairwise comparisons for STAT1α/β, 11 showed lower staining intensity in tumor cells than in AS (AS > tumor), 4 showed approximately equal staining in both compartments (AS = tumor), and 1 showed a greater amount of staining in tumor cells compared with AS (AS < tumor; Table 1). Of 15 comparisons for STAT2, there were 10 AS > tumor, 4 AS = tumor, and 1 AS < tumor. Of 15 comparisons for p48, there were 12 AS > tumor and 3 AS = tumor. Two additional antibodies for STAT1α/β, one rabbit polyclonal antibody and one mouse monoclonal antibody, were tested on several duplicate samples, and all produced identical patterns of staining (data not shown).

Expression of both STAT3α and STAT3β is highest in but not exclusive to the basal cells of the adjacent epithelium, suggesting a possible role for STAT3α/β in maintaining the proliferation state or the suppression of squamous differentia-tion in basal cells (Fig. 2*A*). Of 12 comparisons each for STAT3α and STAT3β, there were 10 AS > tumor and 2 AS = tumor (STAT3α) and 8 AS > tumor and 4 AS < tumor [STAT3β (Table 1)].

The specificity of all of the STAT1α/β, STAT2, p48, and STAT3α antibodies was further verified by Western blotting. All antibodies detected either a single band of the correct size or, in the case of the STAT1α/β antibody, two bands of the correct size corresponding to the α and β STAT1 splice iso-forms (data not shown).

Several Protein Mediators of Type I IFN Signaling Appear to Be Suppressed in Tumor Tissue. For the majority of samples, the expression of one or more of the ISGF3 proteins was reduced in SCCs compared with adjacent nonmalignant epidermal cells (Fig. 1; Table 1). STAT3α and STAT3β were also reduced in tumor tissue as compared with adjacent non-malignant tissue (Fig. 2*A*; Table 1). It should be noted that for

Table 1   Comparison of expression levels of IFNα/β signaling proteins in SCC and nonmalignant AS

The relative expression levels the ISGF3 proteins (STAT1α/β, STAT2, and p48), STAT3α, and STAT3β were scored visually on a scale of 1–4. Samples having a higher score in nonmalignant AS than in tumor (AS > T), equal scores in both (AS = T), or lower scores in AS than in tumor (AS < T) are indicated. Comparisons between AS and tumor tissue were made for contiguous tissues on the same slide and include samples of either primary SCC and AS (14 specimens from 11 patients) or metastases and overlying AS (2 specimens from 2 patients). Note that for patients 2, 3, and 9, two sections from different paraffin-embedded samples of the same tumor were stained. Due to variations in tumor biopsy sizes, the number of available sections was limited; therefore, it was not possible to probe all samples with each antibody. ND, not determined.

Patient no.	Tumor type	Sample	STAT1α/β	STAT2	p48	STAT3α	STAT3β
1	Primary SCC	A	AS = T	AS > T	AS > T	AS > T	AS = T
2	" "	A	AS > T	ND	ND	AS > T	AS > T
	" "	B	AS < T	AS < T	AS > T	AS = T	AS > T
3	" "	A	AS > T	AS > T	AS > T	AS > T	AS > T
	" "	B	AS > T	AS = T	AS > T	AS > T	AS = T
4	" "	A	AS = T	AS > T	AS > T	AS > T	AS > T
5	" "	A	AS = T	AS = T	AS = T	ND	ND
6	" "	A	AS = T	AS = T	AS = T	ND	ND
7	" "	A	AS > T	AS > T	AS > T	ND	ND
8	" "	A	AS > T	AS > T	AS = T	AS > T	AS = T
9	" "	A	AS > T	AS > T	AS > T	ND	ND
	" "	B	AS > T	AS = T	AS > T	AS > T	AS > T
10	" "	A	AS > T	AS > T	AS > T	AS > T	AS > T
11	" "	A	AS > T	AS > T	AS > T	AS > T	AS = T
12	Metastasis	A	AS > T	AS > T	AS > T	AS > T	AS > T
9	Metastasis	A	AS > T	AS > T	AS > T	AS = T	AS > T

most patients, there was a reduction in expression of only a subset of the proteins in tumor tissue (Table 1).

To provide further support for our finding that the ISGF3 and STAT3α/β proteins are less abundant in tumor tissue, we have quantitated the antibody staining intensity on several slides by densitometry. Digital images of immunostained specimens, as shown in Figs. 1 and 2, were taken, and the average sum of pixel intensities for a given area was determined for three measurements of tumor and adjacent nonmalignant epithelium for each slide (Fig. 3). The comparison of the average intensity values for tumor and adjacent epithelial tissue coincided with visual observations. For example, the quantitation for the specimens shown in Fig. 2*B* is shown in Fig. 3*A*.

Table 1 summarizes the relative protein expression levels determined by visual scoring for all pairwise comparisons between adjacent nonmalignant epithelium and tumor tissue on the same slide. Cases for which the expression level of the indicated protein in nonmalignant epithelium is greater than (AS > tumor), equal to (AS = tumor), or less than (AS < tumor) the levels in adjacent tumor cells are indicated.

SCC metastases expressed reduced amounts of STAT1α/β, STAT2, p48, and STAT3α compared with overlying skin (two of two cases for all four proteins), whereas STAT3β was reduced in one of two cases (Fig. 2*B*; Table 1).

Discussion

This study reports the first study of the expression of the ISGF3 (STAT1α/β, STAT2, and p48) and STAT3α/β mediators of type I IFN signaling in human skin. Our findings suggest a role for type I IFN signaling in skin SCC development and progression. Earlier studies have shown that IFN-α and IFN-β can suppress the proliferation of keratinocytes (10), and, more recently, a requirement for STAT1 activation for the antiproliferative effects of IFN-α as well as the apoptotic effect of tumor necrosis factor α has been demonstrated (11, 12). A suppression of STAT1 protein in SCC cells may therefore reflect a loss of both the normal control of proliferation and regulation of apoptosis. However, a simple inverse relationship between STAT1 levels and proliferation may not be universal, because in UT-7/GM leukemia cells and in

leukemic cells from patients, activated STAT1, along with other activated STATs, suppressed differentiation and increased proliferation, respectively (13, 14).

Our finding that STAT3α/β levels are reduced in SCC tumors is somewhat paradoxical when viewed in the context of *in vitro* studies that have established a requirement for STAT3 activation for oncogenic transformation by v-*src* and other oncogenes (15, 16). STAT3 is also constitutively activated in other human tumors and tumor cell lines and has been shown to act as an oncogene (16, 17). In fact, we have observed a constitutive activation of STAT3 in cultured cutaneous SCC cell lines that is not seen in normal human epidermal keratinocytes.[4] However, recent experiments with murine keratinocytes demonstrated an activation of STAT3 during keratinocyte differentiation and also showed that STAT3 DNA binding activity decreased during mitogenic stimulation and reaccumulated as cells entered quiescence (18). Those findings, taken together with our *in vivo* observations, are consistent with the idea that a loss of STAT3 could result in a loss of differentiation and growth control, leading to tumorigenesis. The discrepancy between the *in vitro* findings and our *in vivo* observations is difficult to explain. Given that STAT3 can mediate multiple potentially competing signaling pathways, it may be that in some cell types or environments, STAT3 is acting primarily to stimulate proliferation and/or suppress differentiation, whereas in others, it is doing the opposite.

The work of several investigators has shown that type I IFNs can suppress the tumorigenic phenotype *in vitro* and in nude mice, and at least part of this effect is due to suppression of angiogenesis (Refs. 19 and 20 and the references therein). Additional studies on human epidermal cells have indicated that IFN-β expression is highest in differentiated, nondividing cells and that SCC cells express lower levels of IFN-β than normal skin (21), suggesting that IFN-β acts as a suppressor of proliferation and angiogenesis. Our findings support such a role

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[4] J. L. Clifford, D. G. Menter, X. Yang, E. Walch, M. Wang, T. Schaefer, and S. M. Lippman, unpublished results.
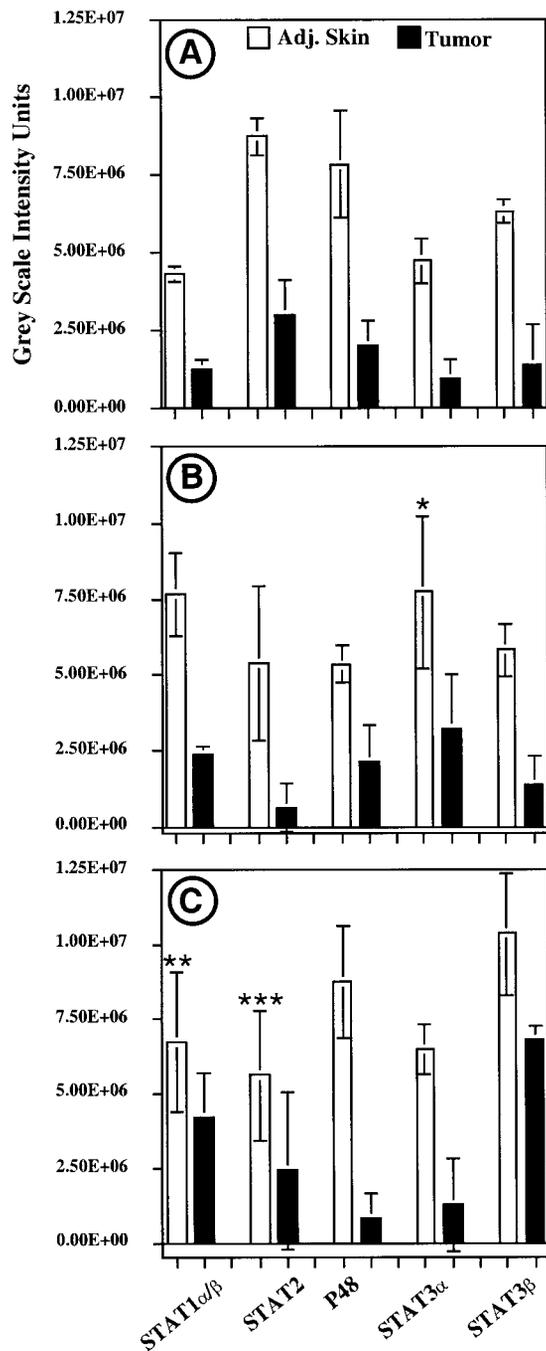
Fig. 3. Densitometric quantitation of antibody staining. Slide images were captured and quantitation was performed as described in "Materials and Methods." *A* and *B* each represent a set of slides probed with antibodies for the indicated proteins (*below C*) for two different biopsy specimens. *C,* STAT1, STAT2, and p48 columns are for a different biopsy specimen than the STAT3α and STAT3β columns. Statistical analysis was performed using a two-tailed Student's *t* test. $P < 0.05$ for all comparisons between AS and tumor samples, except for the indicated pairs: *, $P = 0.065$; **, $P = 0.187$; and ***, $P = 0.180$.

for type I IFNs and further suggest that a decrease in IFN responsiveness may similarly lead to tumorigenicity. The success of IFN-based therapy may be due at least in part to the ability of excess IFN to overcome this reduced responsiveness.

This study is the first to report reduced levels of type I IFN

signaling proteins in human solid tumors of the skin or any other site. We plan future studies of the activated, tyrosine-phosphorylated forms of type I IFN signaling proteins (antibodies for STAT1 and STAT3 have recently become available) that should further our understanding of the function of type I IFNs in skin SCC and nonmalignant tissue.

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