Tumor immune escape by the loss of homeostatic chemokine expression


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The novel keratinocyte-specific chemokine CCL27 plays a critical role in the organization of skin-associated immune responses by regulating T cell homing under homeostatic and inflammatory conditions. Here we demonstrate that human keratinocyte-derived skin tumors may evade T cell-mediated antitumor immune responses by down-regulating the expression of CCL27 through the activation of epidermal growth factor receptor (EGFR)–Ras–MAPK-signaling pathways. Compared with healthy skin, CCL27 mRNA and protein expression was progressively lost in transformed keratinocytes of actinic keratoses and basal and squamous cell carcinomas. In vivo, precancerous skin lesions as well as cutaneous carcinomas showed significantly elevated levels of phosphorylated ERK compared with normal skin, suggesting the activation of EGFR–Ras signaling pathways in keratinocyte-derived malignancies. In vitro, exogenous stimulation of the EGFR–Ras signaling pathway through EGF or transfection of the dominant-active form of the Ras oncogene (H-RasV12) suppressed whereas an EGFR tyrosine kinase inhibitor increased CCL27 mRNA and protein production in keratinocytes. In mice, neutralization of CCL27 led to decreased leukocyte recruitment to cutaneous tumor sites and significantly enhanced primary tumor growth. Collectively, our data identify a mechanism of skin tumors to evade host antitumor immune responses.

The important role for T cells in tumor rejection and their cytolytic activity against tumor cells has been known for decades (1, 2). T cells circulating in the peripheral tissues are constantly screened for foreign and changing self-antigens expressed by malignant cells (3). Subsequently, malignant cells are recognized and eliminated by tumor-associated antigen-specific T cells thereby preventing tumor progression. Although transformed cells frequently express antigens recognizable by the host immune system, established tumors commonly “escape” the immune recognition. An increasing body of literature identifies immune evasion as a critical step in tumor progression (4).

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common malignancies in the Caucasian population (5). The effect of the immune system on the development of skin tumors has been demonstrated by follow-up studies showing that 40% of transplant patients taking immunosuppressants such as cyclosporine, tacrolimus, or azathioprine develop premalignant or malignant skin tumors such as actinic solar keratoses, SCCs and BCCs within 5 years of receiving their transplant (6). The observation that organ transplant recipients treated with preferentially T cell-targeting immunosuppressants have an estimated 65-fold higher risk of SCC and a 10-fold higher risk of BCC development suggests a pivotal role for T cell-dependent immune surveillance in controlling skin tumor progression.

Organ-specific homing of leukocytes is critically regulated by small cytokine-like soluble proteins called chemokines under both homeostatic and inflammatory conditions (7). Recently, it was shown that the novel skin-associated chemokine CCL27/CTACK is specifically expressed in epidermal keratinocytes and mediates the recruitment of skin-homing CCR10+ memory T cells to cutaneous sites (8, 9). Moreover, CCL27 has been associated with the recruitment of specific T cell subsets to the skin during immune surveillance (10, 11).

Although chemokines are likely to play an important role in regulating effector cell recruitment to tumor tissues, our understanding of the biological relevance of chemokines in antitumor immunity is still limited (12). Here we demonstrate that human skin tumors evade T cell-mediated antitumor immune responses by down-regulating the expression of the skin-specific homeostatic chemokine CCL27 through the activation of epidermal growth factor receptor (EGFR) and Ras signaling pathways. We propose that the interference of tumor cells with the recruitment of “surveillance” T cells to sites of tumor progression by down-regulating homeostatic CCL27 expression represents a unique tumor immune escape mechanism.

Results

Loss of the Homeostatic Keratinocyte-Derived Chemokine CCL27 During Cutaneous Carcinogenesis. Because homeostatic leukocyte trafficking is thought to play an important role in the maintenance of the immune surveillance of tissues, we sought to investigate chemokine expression in tissue samples representing different stages of cutaneous carcinogenesis. Quantitative PCR (qPCR) analysis of healthy skin (n = 18), actinic keratoses (premalignant, n = 16), BCCs (semmalignant, n = 23), and SCCs (malignant keratinocyte-derived skin tumors, n = 12) showed a significant (P < 0.001) decrease in CCL27 expression...
in transformed tissues. Although CCL27 mRNA was present at high levels (17.1 ± 13.5 fg/25 ng of cDNA) in healthy skin, its amount was significantly decreased in actinic keratoses (3.3 ± 6.4 fg/25 ng of cDNA; 5-fold reduction; P < 0.001), BCCs (0.6 ± 0.5 fg/25 ng of cDNA; 28-fold reduction; P < 0.0001), and SCCs (0.1 ± 0.2 fg/25 ng of cDNA; 171-fold reduction; P < 0.001), suggesting the loss of CCL27 expression during neoplastic transformation of keratinocytes (Fig. 1A).

Immunohistochemical analysis of healthy skin samples (n = 12) and tissue samples obtained from actinic keratoses (n = 19), BCC (n = 12), and SCC (n = 17) showed that CCL27 was abundantly expressed in normal human skin but showed markedly decreased expression in actinic keratoses, frequently considered as an early stage in the biological continuum that finally leads to the development of SCCs [Fig. 1B and C supporting information (SI) Fig. 5]. CCL27 expression was markedly reduced to absent in BCC and SCC tissue samples, suggesting the progressive loss of CCL27 expression during malignant transformation of keratinocytes (Fig. 1D and E). It is noteworthy that in contrast to transformed cells, the neighboring healthy keratinocytes still expressed CCL27, suggesting the presence of a tumor-specific pathway responsible for the down-regulation and loss of CCL27 protein expression (Fig. 1A and C and D and SI Fig. 5). Quantitative image analysis of immunohistochemical stainings showed that CCL27 protein expression significantly decreased from 20.43 ± 7.74% area in healthy skin specimens to 5.59 ± 3.69% area in BCCs (P < 0.05; Mann–Whitney U test) and to 0.06 ± 0.02% area in SCCs (P < 0.001; Mann–Whitney U test) (Fig. 1F). Moreover, the expression of CCL27 protein was 38% lower in actinic keratoses compared with healthy skin, although this change was not statistically significant. Taken together, analyses of CCL27 mRNA and protein expression uniformly showed the progressive loss of the skin-specific T cell-homing signal CCL27 during cutaneous carcinogenesis.

Activation of Ras Signaling Suppresses CCL27 Expression in Keratinocytes. Activating mutations in the Ras oncogene or alterations in upstream or downstream signaling pathways are frequently present in premalignant and malignant skin neoplasia such as actinic keratoses, SCCs, and BCCs, suggesting its importance in cutaneous carcinogenesis (13–15). To get insights into the regulation of CCL27 expression in keratinocyte-derived tumors, we analyzed the expression of CCL27 in HaCaT cell lines transfected with the activated H-RasV12 oncogene (Fig. 2A–D) (16, 17). Results of the GTP-binding assay demonstrated increased Ras activity in cell lysates of H-RasV12-transfected cell lines compared with untransfected cells (Fig. 2A and B). To investigate the activation status of downstream elements of the Ras pathway in cell lines transfected with H-RasV12, we analyzed the phosphorylation of the extracellular signal-related kinases 1 and 2 (ERK1/2). Immunoblot analysis showed the increased phosphorylation of ERK1/2 in cell lines with high Ras activity, suggesting the constitutive activation of the Ras–MAPK pathway in Ras-transfected keratinocytes (Fig. 2A and B). Examining CCL27 expression with qPCR, we observed that untransfected HaCaT cells showed the highest CCL27 mRNA level, whereas its expression was significantly (P < 0.01, Mann–Whitney U test) decreased in II4, II4RT, ASRT1, and in ASRT3 cell lines (Fig. 2C). Importantly, H-RasV12–HaCaT clones with the highest Ras activity and the highest ERK1/2 activation showed the lowest expression of CCL27 mRNA and protein levels (Fig. 2A–D). Statistical analysis confirmed a strong negative correlation (Pearson’s correlation coefficient = −0.951, P < 0.005) between Ras activity (Fig. 2B) and CCL27 protein levels (Fig. 2D). Contrary to CCL27, the expression of CXCL8, a chemokine recently identified as an important molecule in Ras-induced tumor growth and angiogenesis (18), was up-regulated in H-RasV12–HaCaT clones (data not shown).

We next sought to investigate whether the Ras oncogene is involved in the regulation of CCL27 in primary keratinocytes. To this end, we transiently transfected primary human keratinocytes with an expression plasmid harboring the activated Ras oncogene (H-RasV12), which resulted in detectable expression of the mutant protein as determined by Western blotting (Fig. 2E). Expression of H-RasV12 after transient transfection resulted in a marked decrease of CCL27 expression compared with mock or untransfected keratinocytes (Fig. 2F). In accordance with previous reports demonstrating the induction of CXCL8 expression by activated Ras in several cancer cell lines (18), oncogenic Ras induced the expression of CXCL8 in primary keratinocytes (Fig. 2F). Overall, these results indicate that oncogenic Ras suppresses CCL27 but at the same time induces CXCL8 expression in keratinocytes.

EGFR Signaling Regulates CCL27 Expression in Keratinocytes. Over-expression or constitutive activation of EGFR, an activator of Ras signaling, had been frequently detected in skin tumors including BCCs and SCCs (19). Here, we investigated the role of EGFR signaling in the regulation of CCL27 expression in keratinocytes (Fig. 3). Treatment of human primary keratinocytes...
cytes with recombinant human EGF triggered the phosphorylation of ERK (Fig. 3A) and decreased CCL27 expression (Fig. 3B). Pretreatment with the specific MAPK inhibitor (U0126) abolished ERK phosphorylation and prevented the suppression of CCL27 expression in keratinocytes, demonstrating that ERK plays an important role in the regulation of CCL27.

Next, we investigated whether blockade of EGFR signaling up-regulates CCL27 expression in keratinocytes. To this end, we analyzed the effect of PD168393, a selective and irreversible small molecule EGFR tyrosine kinase inhibitor, on CCL27 expression of cultured primary keratinocytes (Fig. 3C). In these cells, EGFR was constitutively active at a basal level because of the presence of EGF in the standard cell culture medium as demonstrated by Western blot analysis with specific antibodies to phospho-EGFR (data not shown). Blockade of EGFR signaling by treatment of keratinocytes with PD168393 significantly (P < 0.05) induced CCL27 mRNA expression compared with untreated keratinocytes, demonstrating that EGFR signaling regulates CCL27 expression (Fig. 3C).

Tumor patients treated with small-molecule EGFR tyrosine kinase inhibitors such as ibrass or erlotinib frequently develop inflammatory skin lesions (Fig. 3D) (20). To assess the influence of EGFR signaling on CCL27 expression in vivo, we investigated CCL27 protein expression in nonlesional and lesional skin of patients treated with erlotinib, a small molecule inhibitor of EGFR (Fig. 3E). In accordance with the in vitro data, immunohistochemical analysis of skin sections revealed increased CCL27 protein expression in nonlesional skin of patients (Fig. 3E). Importantly,
expression of CCL27 was up-regulated in lesional skin of EGFR inhibitor-treated patients compared with nonlesional or healthy skin samples. CCL27-positive cells were localized to the basal and suprabasal layers of the epidermis (Fig. 3E). These findings suggest that inhibition of EGFR signaling up-regulates CCL27 expression in keratinocytes in vivo and in vitro.

To examine the activation status of the EGFR–Ras–MAPK pathway during skin carcinogenesis, we analyzed the phosphorylation of ERK1/2 in serial sections of skin specimens obtained from healthy skin (n = 7) as well as from actinic keratoses (n = 9), BCCs (n = 11) or SCCs (n = 17) (SI Fig. 6). Quantitative computer-assisted image analyses of immunohistochemical stainings demonstrated a significant increase in the expression of phospho-ERK (p-ERK1/2) in skin tumor specimens. The ratio of p-ERK1/2 expression to that of total ERK1/2 increased 9-fold in BCCs (P < 0.001; Mann–Whitney U test) and 9-fold in SCCs (P < 0.001; Mann–Whitney U test) (SI Fig. 6). Additionally, there was a >80% increase in p-ERK1/2 to total ERK1/2 ratio in actinic keratosis specimens compared with healthy tissues (SI Fig. 6). These results suggest that the MAPK pathway is activated in the majority of skin tumors.

Neutralization of CCL27–CCR10 Interactions Enhances Tumor Progression in Vivo. Homeostatic expression of CCL27 in the skin is aimed at the constitutive recruitment of peripheral immune surveillance T cells into the skin, which enables them to scan the tissue for the presence of nonself-antigens (11). Under homeostatic conditions, only small subsets of CD4 or CD8 cells express CCR10 in the circulation or in spleen. However, in skin-draining lymph nodes the frequency of CD4+CCR10+ and CD8+/CCR10+ lymphocytes was significantly increased by 2- to 3-fold (P < 0.05, Mann–Whitney U test; SI Table 1). Furthermore, single-cell suspensions of murine skin demonstrated the enrichment of skin-infiltrating CD4+ or CD8+ T cells that express CCR10 under homeostatic conditions. Taken together, these observations suggest a role for CCR10+ leukocyte subsets in the skin immune system (SI Table 1).

Injection of murine B16/F10 tumor cells into the dermis of mice represents a well established syngeneic tumor model that induces skin tumors displaying a marked peritumoral infiltrate consistent of T lymphocytes and dendritic cells (21). Using this tumor model, we investigated whether the neutralization of CCL27 in the tumor microenvironment favors tumor progression, and we analyzed the growth of B16/F10 tumors in the presence or absence of CCL27-neutralizing antibodies. As illustrated in Fig. 4A, CCL27-neutralizing antibody treatment significantly (P < 0.05) enhanced tumor growth compared with isotype-treated animals. Histological evaluation of the skin tumors indicated that the CCL27-neutralizing antibody inhibited the recruitment of leukocytes to sites of tumor formation (Fig. 4B). Immunofluorescence analyses confirmed these observations, showing a marked decrease of CD4+CCR10+ and CD8+/CCR10+ lymphocytes in the marginal zones of B16/F10 tumors (SI Fig. 7).

In accordance with these results, qPCR analyses of pooled tumors from animals treated with CCL27-neutralizing antibody compared with the isotype-treated group revealed the decreased expression of T cell markers (TCRβ, CD4, CD8) and T cell-derived mediators (IFN-γ, CCL5, CXCL9), suggesting the decreased infiltration of tumors by T cells (Fig. 4C). Importantly, we observed a marked decrease in the expression of CCR10, the receptor for CCL27, in tumors derived from the neutralizing antibody-treated animals compared with isotype-treated mice (Fig. 4C). In contrast, the increased expression of genes associated with tumor progression, including matrix metalloproteinase 2, 8, and 9 and cyclooxygenase 2, was found in tumors derived from αCCL27-treated animals (Fig. 4C).

Moreover, qPCR analyses of single tumors demonstrated the significant decrease of CD4 and CD8 transcripts in αCCL27-treated compared with isotype-treated tumors (Fig. 4D). In addition, IFN-γ mRNA expression was significantly suppressed within B16/F10 tumors after CCL27 neutralization (Fig. 4D).
Next, we determined tyrosinase mRNA expression in tumor-draining inguinal lymph nodes to estimate B16/F10 tumor burden and lymph node metastasis. Although normal inguinal lymph nodes did not express tyrosinase, mice intradermally injected with B16/F10 cells and receiving sCCL27 had significantly increased tyrosinase expression in tumor-draining lymph nodes compared with isotype-treated controls (Fig. 4E). These observations demonstrated that neutralization of CCL27 results in decreased numbers of tumor-infiltrating CD4 and CD8 lymphocytes, impaired effector cytokine (IFN-γ) production, and enhanced tumor growth as well as lymph node metastasis in an in vivo skin tumor model.

Discussion

BCC and SCC of the skin represent the most common malignancies in the Caucasian population with a total of 1.3 million new cases in the year 2000 in the United States alone, posing a significant threat to public health (according to the American Cancer Society; available at www.cancer.org,2001). Organ transplant patients have a higher incidence of skin cancer and tend to have more aggressive individual lesions, larger numbers of tumors per patient, onset at younger ages, and increased rates of metastasis (22), supporting the idea that the ability of skin tumors to evade immune surveillance represents a central event in tumor progression. Here we report the progressive loss of the homeostatic and skin-associated chemokine CCL27 during malignant transformation of keratinocytes, representing a mechanism of tumor cells to evade from the immune system.

Although tumors of the epidermis may also use other mechanisms that protect them from host immune responses, our studies indicate a prominent role for the down-regulation of CCL27 expression as a mechanism of immune escape.

Ras signaling is altered in many human cancers because of the mutational activation of Ras protooncogene or alterations in upstream or downstream signaling pathways (23). Although most reports associated the Ras oncogene with its ability to prompt tumor growth and survival, acting within the cancer cell, this viewpoint is now changing to take into account data showing that activated Ras regulates chemokine expression (18). In a recent report, Sparmann and Bar-Sagi demonstrated that activated Ras can also induce CXCL8 expression in tumor cells and enhances their tumorigenic potential in a non-cell autonomous manner through stimulation of tumor angiogenesis (18). Here we demonstrate the ability of activated Ras to down-regulate CCL27 expression in skin tumor cells, which adds a quality of oncogenic features to activated Ras such as the regulation of the escape of tumor cells from immune surveillance.

The observation that the inhibition of EGFR tyrosine kinase up-regulates but activated EGFR or activated Ras suppresses CCL27 expression in keratinocytes suggests that EGFR signaling is involved in the regulation of CCL27 expression. Interestingly, one of the major side effects of EGFR inhibitors in cancer patients is the development of severe skin inflammation (20). In line with our in vitro findings, the skin of patients treated with the EGFR inhibitor erlotinib showed markedly increased amounts of CCL27 protein within the epidermis, providing in vivo evidence that CCL27 expression is regulated by EGFR signaling. Previously, we could show that CCL27–CCR10 interactions play an important role in T cell-mediated skin inflammation (9). Thus, elevated CCL27 production induced by the systemic treatment of cancer patients with EGFR inhibitors together with other nonspecific stimuli may play a role in the development of erlotinib-mediated skin inflammation.

An interesting phenomenon relating to viral immune escape is that human herpes virus 8, implicated in the pathogenesis of Kaposi’s sarcoma, encodes a chemokine-homolog (vMIP-II) with an antagonistic activity against different chemokine receptors, which inhibits the recruitment of effector cells to the sites of viral replication (24). The observation that vMIP-II dramatically reduces T cell-mediated inflammation in a model for virus-induced inflammation strongly suggests that modulation of chemokine signals represents an important and efficient mechanism for evading host immune responses (25). Our results show that similar to viruses, tumor cells may target chemokine-driven leukocyte recruitment pathways to escape immune surveillance. In vivo, neutralization of CCL27–CCR10 interactions resulted in decreased leukocyte recruitment and the significant enhancement of primary tumor growth.

Complementary to our findings, Okada et al. (26) recently demonstrated that overexpression of CCL27 in melanoma cells resulted in impaired tumor growth because of the recruitment and accumulation of T cells and natural killer cells at tumor sites resulting in enhanced antitumor immune responses. Moreover, when CCL27 was transfected into ovarian carcinoma cells, the transfected tumor cells overexpressing CCL27 either had a decreased growth rate or were rejected because of enhanced antitumor immune responses (27). The antitumor activity induced by CCL27 was T cell-dependent, involving both CD4+ and CD8+ T cells (28). These observations strongly argue for an important role for CCL27 in maintaining skin homeostasis.

Collectively, our results suggest a mechanism that may enable transformed keratinocytes to evade the host immune response through down-regulating the homeostatic expression of CCL27. Because homeostatic CCL27 expression is implicated in the basal T cell trafficking during immune surveillance, the progressive loss of CCL27 expression results in the decreased immunological control over the tissue. Our findings suggest that activating mutations in the Ras oncogene and EGFR signaling contribute to the progressive loss of CCL27 production in keratinocyte-derived malignancies. Thus, our findings identify the progressive loss of homeostatic chemokine expression as a pathway of tumor immune escape and suggest strategies for antitumor immunotherapy.

Materials and Methods

Cell Culture and Transient Transfection. HaCaT keratinocytes and H-RasV12-transfected HaCaT clones were cultured as described previously (17). Human primary epidermal keratinocytes were purchased from Cambrex and cultured as described (8). Cells were treated with 10 ng/ml TNF-α and 5 ng/ml 1β (R&D Systems) for 4 h or left untreated. Primary human keratinocytes were transfected with an activating mutant of Ras (H-RasV12) in the expression vector pDCR (a gift from Craig Webb, Frederick, MD) as described by Tscharnkte et al. (29).

Patients. Skin biopsies were taken, after obtaining informed consent, from either healthy individuals undergoing plastic surgery or patients suffering from actinic keratoses, BCCs, or SCCs. The clinical diagnosis was confirmed by histological evaluation. Moreover, skin biopsies were collected from patients receiving therapy with the EGFR antagonist erlotinib. All studies were approved by the appropriate ethics committees.

Quantitative Real-Time PCR (TaMan) Analysis. qPCR analysis was performed as described previously (8). Primers and target-specific probes were obtained from Applied Biosystems. Gene-specific PCR products were measured by means of an ABI PRISM 7700 or 5700 sequence detection system (Applied Biosystems). Target gene expression was normalized to the expression of 18S rRNA or ubiquitin.

Immunohistochemistry. Skin sections were fixed and stained with monoclonal antibodies against human CCL27 (R&D Systems), total ERK, P-ERK (Cell Signaling) or isotype controls (Jackson...
ImmunoResearch Laboratories) as described previously (8, 9). Images were acquired by using a Zeiss Axiovert2 MOT microscope, Zeiss AxioCam MRc with Zeiss AxioVision 4.4 software. For the quantification of CCL27 and p-ERK stainings, images taken from sections were saved in an uncompressed TIF format with the AxioVision 4.4 software and exported to Adobe Photoshop 7.0 (Adobe Systems, Inc.). Three representative regions of interest were drawn in a frame (100 × 110 pixels each) and the percent of the area stained was measured by using the ANALYZE/MEASURE tool of ImageJ (National Institutes of Health).

Ras Activity Assay, Antibodies, and Immunoblotting. The activity of wild-type and constitutive active Ras was determined by using the EZ-Detect Ras activation kit as described by the manufacturer (Pierce Biotechnology). Cells were lysed in Nonidet P-40-containing buffer [25 mM Tris·HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT, 5% (vol/vol) glycerol], and cytoplasmic cell extracts were isolated by high-speed centrifugation. Ras protein was detected by staining with an anti-Ras mouse monoclonal antibody (Pierce) and visualized by using the ECL chemiluminescence system (Amersham Biosciences). For the detection of ERK phosphorylation, cytoplasmic extracts were analyzed by immunoblotting with antibodies (Cell Signaling) against phospho-ERK1/2 (p44/42 MAPK; 9106) and ERK1/2 (9102).

Statistical Analysis. Statistical significance was determined by the Mann–Whitney U test with the SPSS software (version 12.0). Correlation between the expressions of different genes in the same samples was made by using Pearson's correlation test with SPSS software.

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