Mechanisms Underlying Imiquimod-Induced Regression of Basal Cell Carcinoma In Vivo

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Background: Imiquimod is a local immune response modifier that has demonstrated potent antiviral and antitumor activity. It enhances innate and acquired immune responses via endogenous cytokine production and has proven efficacious in clearing superficial basal cell carcinoma (sBCC).

Objective: To evaluate the mechanisms by which topical imiquimod treatment leads to sBCC clearance in vivo.

Design: A pilot, open-label, nonrandomized study.

Setting: Zurich, Switzerland.

Patients: Six persons 18 years or older who had nonrecurrent primary tumors that had not undergone previous biopsy or treatment but were suitable for treatment by surgical excision. The tumors were located on the scalp, extremities, or trunk; had a minimum diameter of 1 cm and a maximum diameter of 2 cm; and were clinically and histologically consistent with sBCC.

Interventions: Daily application of 5% imiquimod cream 5 times per week for a maximum of 6 weeks. When the tumor began to show signs of erosion, it was surgically excised.

Outcome Measures: Parameters reflecting tumor apoptotic status (Bcl-2), expression of death receptors (Fas and Fas ligand [FasL]), intercellular adhesion molecule (ICAM) 1, immunosuppressive microenvironment (interleukin 10), and antigen presentation machinery (transporter associated with antigen presentation [TAP] 1) before and after imiquimod treatment were evaluated. The changes in the interferon γ messenger RNA (mRNA) levels relative to CD4 and CD8 mRNA were assessed using quantitative polymerase chain reaction.

Results: Tumor cells became more susceptible to apoptosis through decreased Bcl-2 expression after treatment with 5% imiquimod cream. Inflammatory infiltrate developed rapidly (within 3 to 5 days after treatment initiation) and was associated with the enhanced expression of ICAM-1. This early response tended to be a mixed cellular response of macrophages and lymphocytes. Interferon γ was produced by CD4 and CD8 T cells. Imiquimod treatment induced a massive increase in macrophage peritumoral and intratumoral infiltration. Interleukin 10 was produced by infiltrating cells but was not produced by tumor cells. Tumor expression of TAP-1 and Fas/FasL appeared to be unaffected in the first 5 days of treatment.

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Imiquimod (1-(2-methylpropyl)-1H-imidazo[4,5-C]quinolin-4-amine) belongs to the group of imidazoquinolines, synthetic local immune response modifiers that have demonstrated potent antiviral and antitumor activity as well as effects on immunologic memory. Imiquimod induces migration and activation of skin antigen-presenting (Langerhans) cells, as well as the production of local cytokines such as interferon (IFN) α, tumor necrosis factor (TNF) α, interleukin (IL) 1α, and IL-12, resulting in an enhanced innate immune response. This in turn leads to activation of naïve TH cells into TH1 cells, which are potent producers of IFN-γ, thus indirectly leading to the enhancement of acquired immunity. The stimulation of innate and acquired immunity may finally lead to regression of viral-induced lesions and neoplasms. Previous studies have shown that imiquimod-induced wart regression was associated with induction of the cytokines listed above and with cellular infiltrates consistent with a TH1 cell–mediated immune response.

Basal cell carcinoma (BCC) is the most common malignancy in the white human population worldwide, and its incidence has been increasing in the last decade. Basal cell carcinoma tends to grow slowly and rarely metastasizes. Additionally, BCC can spontaneously regress, exhibiting a shift in immune response toward a TH1 profile, which

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in turn suggests that the immune system plays an active role in BCC surveillance. 

Immune response in BCC can be modulated by intralesional injections of interferons or by topical application of imiquimod, each resulting in complete clearance of the tumor. 

Imiquimod has been tested by topical application of imiquimod, each resulting in complete clearance of the tumor. 

We sought to investigate the mechanisms by which topical imiquimod treatment leads to destruction of BCC by taking 4 general considerations into account. First, the tumor cells have an enhanced ability to survive. Overexpression of Bcl-2 has been reported in BCC, and may be one of the ways to inhibit proapoptotic signaling and promote survival without promoting cell proliferation. 

Second, cytokines induced by imiquimod (eg, IL-1, IFN-α, IFN-γ, and TNF-α) have been shown to induce apoptosis in susceptible targets through up-regulation of death receptors. 

Third, the activation of cellular immunity seems to be sufficient to overcome the tumor immune escape strategies. Namely, BCC tumor nests are frequently surrounded by a varying number of mononuclear cells. However, only rare mononuclear cells are seen infiltrating the tumor islets. Although these infiltrating cells are needed for the action of activating markers, tumor recognition and destruction fails to happen. 

One explanation may be in the impaired antigen presentation due to the defective antigen-presenting machinery (eg, transporter associated with antigen presentation [TAP] 1) or in the production of immunosuppressive cytokines such as IL-10 

The fourth category includes factors that may favor tumor destruction, such as the up-regulation of endothelial cell adhesion molecules (such as intercellular adhesion molecule 1 [ICAM-1]) allowing the influx of immunofur citor cells. Therefore, we evaluated the expression of a panel of markers (Bcl-2, Fas, Fas ligand [Fasl], ICAM-1, IL-10, TAP-1, and CD68) in BCC before and after imiquimod treatment by means of immunohistochemical analysis. The changes in the IFN-γ messenger RNA (mRNA) levels relative to CD4 and CD8 mRNA after imiquimod treatment were assessed using real-time quantitative polymerase chain reaction (PCR) methodology. 

Our results suggest that after imiquimod treatment, tumor cells become more susceptible to apoptosis through decreased Bcl-2 expression; the infiltrate develops rapidly (within 3 to 5 days after treatment initiation) and is associated with the enhanced expression of adhesion molecules by vascular endothelium. Such an early response tends to be mixed and consists of macrophages and lymphocytes (CD4 and CD8). Imiquimod treatment induces a massive increase in macrophage peritumoral and intratumoral infiltration; IFN-γ seems to be produced by CD4 and CD8 T cells, and IL-10 is produced by invading inflammatory cells but not by tumor cells. Tumor expression of TAP-1 and Fas/Fasl seems unaffected in the first 5 days of treatment.

**METHODS**

**PATIENTS AND ELIGIBILITY**

This pilot, open-label, nonrandomized study to evaluate the alteration in BCC tumor defenses after treatment with 5% imiquimod cream (Aldara; 3M Pharmaceuticals, St Paul, Minn) sponsored by 3M Pharmaceuticals was approved by the institutional ethics committee. Patients were eligible to participate if they were at least 18 years old and had a primary tumor (not recurrent and without previous biopsy or treatment) suitable for treatment by surgical excision. The tumors were located on the scalp, extremities, or trunk; had a minimum diameter of 1 cm and a maximum diameter of 2 cm; and were clinically and histologically consistent with superficial BCC. Prior to entering the study, all patients provided written informed consent. Six patients completed the study.

At the screening visit, a biopsy specimen (4-mm punch) consisting of no more than 25% of the tumor was taken for histologic confirmation of superficial BCC and evaluation of base-}

**REAL-TIME QUANTITATIVE PCR**

Total RNA was extracted from frozen biopsy specimens using TRIzol reagent (Invitrogen AG, Basel, Switzerland) according to the manufacturer’s recommendations. Approximately 1 µg of total RNA was reverse transcribed using oligonucleotide primers and avian myeloblastosis virus (AMV) reverse transcriptase (1st Strand cDNA Synthesis Kit for RT-PCR [AMV]; Roche Molecular Biochemicals–RMB, GmbH, Mannheim, Germany) at 42°C for 1 hour.

The PCR amplifications were carried out, as previously described, using the following primer sets: CD4 primers (CD4 (forward) 5'-AGCTCTGCATCGTTTTGGGTT-3', IFN-γ (reverse) 5'-GTTCATTATCCGCTACATCTGA-3', CD4 (forward) 5'-CCCTGAGAACCCATCATCTAG-3', CD8 (reverse) 5'-GTGGCGTCGCGCGCCCG-3', CD4 mRNA copy numbers/µL were normalized by the absolute glyceraldehyde-3-phosphate dehydrogenase mRNA copy number/µL. The IFN-γ mRNA copy number was evaluated relative to the CD8 and CD4 mRNA copy numbers, with an immunological assumption that CD8 and CD4 populations accounted for most of the local IFN-γ production.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Paraffin-embedded tissue sections were stained with the following primary antibodies: Bcl-2, Fas, Fas ligand, ICAM-1 (IgG1 mouse mAb; Novocastra Laboratories Ltd, New Castle, England), Fasl (IgM mouse mAb; Novocastra Laboratories Ltd), IL-10 (mouse IgG2b mAb; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and TAP-1 (gift from Soldano Ferrone, MD, PhD, Roswell Park Cancer Institute, Buffalo, NY). Melanoma cryostat and paraffin-embedded tissue sections known to express
changes in infiltrate density and in immunohistochemical markers in basal cell carcinoma following topical imiquimod treatment

**Table**

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Abbreviations: BVs, blood vessels; FasL, Fas ligand; hpf, high-power field; ICAM-1, intercellular adhesion molecule 1; IL-10, interleukin 10; INF, infiltrate; nc, no change; TAP-1, transporter associated with antigen presentation; TU, tumor; +++, more than 50% of the cells/hpf; +, fewer than 50% of the cells/hpf; ±, fewer than 5% of the cells/hpf; −, no expression; ↑, up-regulation; ↓, down-regulation.

*Status after x days* indicates that time when the tumor showed signs of erosion and was excised.

†Observed only on BVs.

IL-10 were used as positive controls. Human testis paraffin-embedded tissue sections were used as positive control for Fas and FasL expression, according to the manufacturer’s recommendation.

After heat antigen retrieval (Target retrieval solution, DAKO), immunohistochemical analysis was performed using alkaline phosphatase-anti-alkaline phosphatase technique. Briefly, 3- to 5-µm-thick tissue sections adherent to slides coated with 0.1% (wt/vol) poly-L-lysine were deparaffinized with xylene and rehydrated. Non-specific binding sites were blocked by incubating slides with normal rabbit serum for 15 minutes at room temperature. Tissue sections were incubated with an excess of mAb for 60 minutes. This was followed by 3 cycles of sequential incubations with rabbit antimouse IgG xenobodies and alkaline phosphatase-anti-alkaline phosphatase complexes. Incubations in the first cycle were of 30-minute duration; the second and third cycles were 10 minutes each. All incubations were performed at room temperature in a moist chamber. The immunoreaction was visualized with a developing solution containing neufuchsin (DAKO). Finally, sections were counterstained with 1% hematoxylin. Histologic sections were analyzed microscopically by independent observers who counted the number of cells labeled with each of the above-mentioned markers in 4 random high-power fields (hpf’s).
Figure 1. The expression of different markers in superficial BCC lesions: CD68 (A, baseline [BL]; B, posttreatment [PT]; Bcl-2 (C, BL; D, PT); Fas (E, BL; F, PT); FasL (G, BL; J, PT); ICAM-1 (H, BL; I, PT); TAP-1 (K, BL; L, PT); and IL-10 (M, BL; N, PT).
weakly baseline positive). Conversely, FasL expression decreased in 3 subjects whose baseline expression was the greatest. Infiltrating cells showed increased FasL immunoreactivity in all cases after treatment with imiquimod 5% cream (Figure 1J). Fas expression, on the other hand, was not demonstrated in any of the subjects’ tumors either at the baseline level (Figure 1E) or after treatment (Figure 1F). An increasing number of infiltrating cells (interestingly among them numerous mast cells) exhibited Fas immunoreactivity after treatment (Table). No ICAM-1 immunoreactivity was detected on tumor cells, either at baseline or after treatment (Table, Figure 1H). On the other hand, ICAM-1 expression on endothelial cells was notably increased after imiquimod treatment in 5 of 6 cases (Figure 1I).

Tumor cells from all subjects were uniformly negative for IL-10 expression at baseline, with a minor increase in 1 subject after treatment (Table, Figure 1M). Infiltrating cells displayed either no or very weak IL-10 immunoreactivity at baseline, which was increased after treatment in all 6 cases (Figure 1N). Tumor cells exhibited either very weak (<5% positive cells/hpf in 5 of 6 subjects) or no TAP-1 expression (Figure 1K). One subject responded with an increase in TAP-1 expression after treatment, while the others showed no change (Figure 1L). Infiltrating cells were consistently TAP-1 positive at baseline, subsequently showing an increase (in 5 of 6 subjects) in TAP-1 immunoreactivity after treatment (Table, Figure 1L).

Five subjects had their biopsies performed 3 to 5 days after treatment, while 1 subject (patient 1) waited 18 days; therefore, it was interesting to look for differences in staining patterns between subjects with shorter and longer treatment duration. In this regard, the subject who waited 18 days demonstrated a greater increase in FasL expression of the posttreatment infiltrating cells and was the only subject to show increased TAP-1 expression in posttreatment tumor cells.

REAL-TIME QUANTITATIVE PCR ANALYSIS

Changes in the level of IFN-γ mRNA relative to CD4 and CD8 mRNA were evaluated using real-time quantitative PCR. This analysis allows an estimation of the relative production of IFN-γ mRNA per cell type based on an estimation of the relative number of CD4 and CD8 cells in the tissue (Figure 2A). The increased numbers of infiltrating cells coincided with several-fold increases in lymphocyte mRNA in 3 subjects. The lymphocyte mRNA decreased while the histologic analysis showed an increase in infiltrating cells in 2 subjects. This could indicate that the infiltrating cells were monocyte macrophages rather than lymphocytes, or it may imply death of lymphocytes. In 4 subjects (patients 1, 3, 4, and 5), CD8 mRNA increased by 3- to 5-fold after treatment. In 3 of those 4 subjects (patients 3, 4, and 5), the same was true for CD4 mRNA (Figure 2A).

The ratio of IFN-γ mRNA to CD8 mRNA increased in 4 of 6 subjects after treatment, and the corresponding ratio to CD4 mRNA increased in 4 of 6 subjects (while 1 subject [patient 1] had a decreased CD8/IFN-γ ratio as well) (Figure 2B). Absolute numbers of IFN-γ mRNA in 4 subjects varied slightly after treatment (an average of 15%) (Figure 2A). On the other hand, 2 subjects demonstrated a substantial increase in absolute IFN-γ copy numbers. In those subjects where the IFN-γ values were essentially unchanged, the increase in either CD8 or CD4 mRNA copy number was responsible for the lower values of the corresponding ratio to IFN-γ. In 2 subjects whose IFN-γ mRNA increased after treatment, CD4 and CD8 copy numbers increased. Half of the subjects demonstrated IFN-γ/CD8 ratio that was greater than the corresponding ratio to CD4 mRNA, and vice versa (Figure 2B). Both CD4 and CD8 cells seemed to produce IFN-γ. CD8 cells seem to be a prominent component of infiltrating cells on imiquimod treatment, taking into account that absolute CD8 copy number increased after treatment in 4 of 6 subjects and that 3 of 6 subjects demonstrated a decrease in CD4/CD8 ratio after treatment (Figure 2B).

COMMENT

Immune response modifiers are substances that directly influence a specific immune function or modify one or more components of the immunoregulatory network to achieve an indirect effect on specific immune function.21 Imiquimod is one such agent with potent antiviral and antitumor activity. Although imiquimod’s mechanism of action is not yet fully understood, its clinical
benefits are likely to be related to its immunomodulatory properties. Application of imiquimod induces a cascade of cytokines (eg, IFN-α, TNF-α, IL-1α, IL-12, and IFN-γ) with profound effects on innate and acquired immunity and on immunologic memory and antigen presentation. Imiquimod is found to be efficacious in clearing superficial BCC. Similar to interferon, imiquimod seems to modulate the immune response in BCC, leading to tumor recognition and destruction. We studied the mechanisms by which topical imiquimod treatment leads to BCC clearance by addressing, in particular, parameters thought to reflect cellular influx and cytokine release (CD68, CD4, CD8, and IFN-γ), tumor apoptotic status (Bcl-2), local expression of death receptors (Fas and FasL) and adhesion molecules (ICAM-1), immunosuppressive microenvironment (IL-10), and antigen presentation machinery (TAP-1).

Basal cell carcinomas are regarded as indolent slow-growing tumors with limited metastatic potential. One of the explanations for the indolent behavior of primarily nonaggressive BCCs (such as superficial BCCs) might be that these tumors strongly express Bcl-2, an antiapoptotic member of Bcl-2 gene family. Aberrant expression might be that these tumors strongly express Bcl-2, an antiapoptotic member of Bcl-2 gene family. Aberrant expression of Bcl-2 in BCC might account for extended tumor cell survival without influencing cell proliferation. Similarly, the importance of these cells in the elimination of tumor cells prior to imiquimod treatment (consistent with the observation of the pre-treatment peritumoral infiltrate). Mackenzie-Wood et al found a similar infiltrate pattern at the baseline level as well as the similar CD4/CD8 ratios. Imiquimod treatment, however, induced a rapid increase in infiltrate density in all subjects studied. This is the first report showing how rapidly the infiltrate develops after imiquimod treatment: 5 out of 6 subjects were evaluated within 3 to 5 days after treatment initiation. This early infiltrate seems to be mixed in nature, containing cells of lymphoid and myeloid origin. Interferon-γ appeared to be produced by both CD4+ and CD8+ T cells, as shown by the quantitative PCR data, even though we cannot exclude the expression of IFN-γ mRNA by other CD4+ cell types such as natural killer cells and monocyte-derived cells.

Minor changes in IFN-γ mRNA were observed in 4 of 6 patients, which were probably due to the short study duration. The greatest increases in IFN-γ, however, were associated with decreased CD4/CD8 ratios. When the posttreatment increase in more than half of the subjects and a reversal of the CD4/CD8 ratio are considered, CD8+ cytotoxic/suppressor T cells tend to represent a substantial part of the infiltrate induced by imiquimod treatment. The observed rapid increase in CD8 mRNA may lead us to speculate that the adaptive immune system may have been aware of the presence of tumor cells prior to imiquimod treatment (consistent with the observation of the pre-treatment peritumoral infiltrate). Mackenzie-Wood et al reported a similar reversal of the median CD4/CD8 lymphocyte ratio in Bowen disease after imiquimod treatment, suggesting the importance of these cells in the elimination of tumor. Moreover, we found that imiquimod treatment resulted in a massive increase in cells of monocyte-macrophage origin (CD68+) surrounding and often infiltrating tumor islets. Macrophages exhibit potent tumoricidal activity on activation (eg, “classic” macrophage stimulant IFN-γ), with the release of toxic factors (TNF-α), serine proteases, and reactive nitrogen and oxygen intermediates, resulting in cell lysis. (Of note, most of these molecules can be induced by imiquimod.) Given the cytokines known to be up-regulated by imiquimod (eg, IL-1α, TNF-α, macrophage inflammatory protein 1α and 1β, macrophage chemotactic protein 1), the attraction of CD68+ cells should be expected. Rapid accumulation of CD68+ cells might actually reflect a quick response to imiquimod, since imiquimod represents a specific ligand for toll-like receptor 7, toll-like
receptor 7 is expressed in plasmacytoid and myeloid dendritic cells, and on its activation, these cells produce IFN-α and IL-12, respectively.

The absence (or the sparsity) of the tumor-infiltrating cells in BCC has been attributed to the lack of expression of adhesion molecules on tumor cells, thus preventing binding of immune cells. ICAM-1 is a cell surface glycoprotein expressed constitutively on a wide variety of cell types, which is up-regulated in response to a number of inflammatory mediators, such as IL-1, TNF-α, and IFN-γ. Previous studies have shown that BCC cells either did not express, or only rarely expressed, ICAM-1. However, ICAM-1 expression by dermal endothelial cells was more intense in the areas of peritumoral inflammatory infiltrate than in normal skin. At baseline, all of the tumor biopsy samples demonstrated no ICAM-1 expression on the tumor and a weak expression on dermal endothelial cells. Imiquimod treatment, however, strongly enhanced the ICAM-1 expression on dermal endothelial cells without affecting the ICAM-1 expression on the tumor cells. The increase in ICAM-1 expression by endothelial cells paralleled the increase in infiltrate density, suggesting that through expression of ICAM-1 on vascular endothelium, imiquimod treatment recruits the cells from the circulation.

Infiltrating cells fail to eliminate the tumor despite the fact that they often express “activation” markers. This might be a result of impaired antigen presentation due to the defect in antigen-presenting machinery (e.g., TAP-1) or in the production of immunosuppressive TGF-β cytokines (e.g., IL-10), resulting in the depletion of antigen-presenting cells, down-regulation of costimulatory molecules, and inhibition of local cellular immunity. Our data showed either very weak or no TAP-1 expression in BCC at the baseline level. Only 1 case responded with an increase in TAP-1 immunoreactivity, whereas the rest of the cases showed no change. To our knowledge, this is the first report on TAP-1 expression in BCC, demonstrating reduced expression of the molecules involved in antigen presentation that could not be induced by imiquimod treatment. Whether this is due to the short treatment duration (1 case, however, did show an increase after almost 3 weeks of treatment) or to other defects leading to IFN-γ unresponsiveness (e.g., defective IFN-γ signaling), shown to be important in TAP-1 induction, remains to be elucidated.

Interleukin 10 is another factor whose production in BCC is thought to account for silencing the cellular immune response. In our study, however, BCC cells were uniformly negative for IL-10 expression at baseline and showed little change after imiquimod treatment. This discrepancy between ours and other studies of superficial BCC is probably owing to the different starting material (cryostat sections) and antibodies and a different method used to detect IL-10. Infiltrating cells demonstrated an increase in IL-10 immunoreactivity after treatment in all cases evaluated. Imiquimod has been reported to induce IL-10 in peripheral blood mononuclear cells in vitro. Interleukin 10 has been identified as one of the key immunomodulatory cytokines capable of mediating both immunosuppressive and immunostimulant effects. Besides immune dampening, IL-10 enhances the proliferative responses of activated CD8+ T cells, can rescue T cells from apoptotic cell death, and can boost the promotion of antitumor CD8+ T cells as well as the maintenance of their effector function outside of the lymph node.

In conclusion, our results demonstrate the changes in BCC tumor biology and peritumoral environment in vivo after immunomodulatory treatment with imiquimod. Tumor clearance is likely to be the result of increased susceptibility to apoptosis and a rapid and massive increase in the number of peritumoral and intratumoral infiltrating cells. Macrophages seem to represent an important part of the imiquimod-induced inflammatory infiltrate in BCC. Interleukin 10 is produced by infiltrating cells but not by tumor cells; tumor expression of TAP-1 and Fas/FasL seems unaffected in the first 5 days of treatment. Larger studies are needed to better define the mechanisms by which topical imiquimod treatment leads to BCC tumor clearance. More insight into these mechanisms will provide a basis for the design of more effective dosing schedules and therapeutic regimens in the treatment of epithelial skin cancer.

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