The Small Antitumoral Immune Response Modifier Imiquimod Interacts with Adenosine Receptor Signaling in a TLR7- and TLR8-Independent Fashion

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Imiquimod, a small-molecule immune response modifier of the imidazoquinoline family, has shown profound antitumoral and antiviral efficacy both *in vitro* and in clinical applications *in vivo*. It has been demonstrated that this activity is mediated through the Toll-like receptor (TLR)7- and TLR8-signaling cascade resulting in the secretion of proinflammatory cytokines and, consecutively, induction of a tumor-directed cellular immune response. In addition, imiquimod exerts a direct proapoptotic activity in tumor cells. We demonstrate here that imiquimod induces activation of the transcription factor NF-κB and the downstream production of proinflammatory cytokines in the absence of TLR7 and TLR8. In Chinese hamster ovary cells stably transfected with the human adenosine receptor subtypes, we then show in radioligand-binding competition experiments that imiquimod binds to adenosine receptors at concentrations relevant in clinical settings, with highest affinities to the A_1 and A_{2A} subtypes. The effect on the receptor-mediated activation of adenylyl cyclase was also studied, and these experiments revealed that imiquimod acts as an adenosine receptor antagonist. In addition, imiquimod had an inhibitory effect on adenylyl cyclase activity downstream from the receptor. Finally, using transformed human keratinocytes, we provide experimental evidence that imiquimod and A_{2A} adenosine receptor-specific compounds similarly induce proinflammatory cytokines in the absence of immune cells. Thus, imiquimod appears to suppress an important feedback mechanism of inflammation by antagonism of adenosine receptor-dependent increase of cAMP and a concomitant receptor-independent inhibition of cAMP production. These novel mechanisms presumably act synergistic with the positive induction of proinflammatory cytokines and can, at least in part, explain the profound inflammation observed in some patients *in vivo*.

Journal of Investigative Dermatology (2006) **126,** 1338–1347. doi:10.1038/sj.jid.5700286; published online 30 March 2006

INTRODUCTION

Small molecules with specific activity against malignant or virally infected cells are attractive candidates with respect to new antitumoral treatment options. Among such compounds, immune response modifiers of the imidazoquinoline family, in particular the lead compound imiquimod, have attracted considerable interest owing to their profound antiviral and antitumoral activities (Stanley, 1999; Marks *et al.*, 2001; Bath-Hextall *et al.*, 2004; Gupta *et al.*, 2004; Schön and Schön, 2004; Urosevic *et al.*, 2004; Burns and Brown, 2005; Schulze *et al.*, 2005). Recent studies have demonstrated elegantly that imidazoquinoline family members exert their biological activity through direct activation of signaling through Toll-like receptor (TLR)7 and/or TLR8 (Hemmi *et al.*, 2002; Jurk *et al.*, 2002; Gorden *et al.*, 2005), both of which have recently been identified as natural receptors for single-stranded RNA (Diebold *et al.*, 2004; Heil *et al.*, 2004). The activity of imiquimod is then mediated, at least in part, through intracellular activation of the transcription factor NF-κB, which upon release from its inhibitor IκB migrates to the nucleus and upregulates transcription of various cytokines including tumor necrosis factor-alpha (TNFα), IL-2, IL-6, IL-12, G-CSF, GM-CSF, IFNγ, and IFNα, as well as chemokines such as IL-8, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and membrane cofactor protein-1 (Miller *et al.*, 1994; Reiter *et al.*, 1994; Weeks and Gibson, 1994; Gibson *et al.*, 1995; Megyeri *et al.*, 1995; Wagner *et al.*, 1997). The net result of these effects is a profound stimulation of a tumor-directed cellular immune response that is seen in tumors of different origin, for

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Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; Cl-IB-MECA, N^6-(2-chloro-3-iodobenzyl)adenosine-5′-N-methyluronamide; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-PCR; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine|TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha

Received 16 October 2005; revised 6 February 2006; accepted 9 February 2006; published online 30 March 2006

example, cutaneous melanomas and nonmelanoma skin cancer (Beutner *et al.*, 1999; Marks *et al.*, 2001; Stockfleth *et al.*, 2001; Bong *et al.*, 2002). Indeed, imiquimod has demonstrated good efficacy in the topical treatment of basal cell carcinomas (Beutner *et al.*, 1999; Hannuksela-Svahn *et al.*, 2000; Marks *et al.*, 2001; Tyring, 2001), cutaneous squamous cell carcinomas *in situ* (actinic keratoses and Bowen's disease) (Stockfleth *et al.*, 2001; Tyring, 2001), and cutaneous metastases of malignant melanoma (Steinmann *et al.*, 2000; Bong *et al.*, 2002; Schön *et al.*, 2004).

Although dendritic cells are thought to be the primary imiquimod-responsive cell type (Miller *et al.*, 1994; Reiter *et al.*, 1994; Burns *et al.*, 2000; Suzuki *et al.*, 2000; Hemmi *et al.*, 2002), imiquimod at higher concentrations also exerts direct proapoptotic activity against various tumor cell populations *in vitro* and *in vivo* (Schön *et al.*, 2003, 2004; Sidbury *et al.*, 2003), and it can induce opioid growth factor receptors in tumor cells (Urosevic *et al.*, 2004).

We report here the surprising observation that the biological activity of imiquimod is broader and more complex than could be discerned from previous reports, inasmuch as the compound induced proinflammatory cytokines in TLR7- and TLR8-negative cells, presumably through interference with adenosine receptor signaling. Given that adenosine receptor signaling is involved in inflammatory cascades (Montesinos *et al.*, 2003; Sitkovsky *et al.*, 2004; Odashima *et al.*, 2005), there may be an imiquimod-induced regulatory loop involving adenosine receptors that augments the inflammatory responses observed *in vivo*.

RESULTS

Induction of proinflammatory cytokines by the small-molecule immune response modifier imiquimod in TLR7- and TLR8-negative cells

To further determine the mode of action of small-molecule immune response modifiers, we cultured various cell lines, such as peripheral blood mononuclear cells, keratinocyte-derived tumor cells, melanoma lines, or leukemia cells, in the presence of imiquimod and studied their cytokine expression profile. Most of the transformed cell lines studied did not express detectable levels of either TLR7 or TLR8 (Figure 1a), the only imiquimod-binding receptors described thus far (Hemmi *et al.*, 2002; Jurk *et al.*, 2002; Gorden *et al.*, 2005). Thus, our results demonstrating that imiquimod induced upregulation of proinflammatory cytokines including TNFα, IL-1β, IL-6, and IL-8 in TLR7- and TLR8-negative cells, such as the keratinocyte-derived cell line HaCaT (Figure 1b, c, and e), was surprising and indicated a biological activity of imiquimod that was exerted independent of TLR7, TLR8, or dendritic cells. Strong induction of both IL-6 and IL-8 mRNA was observed as early as 30 minutes after exposure of the cells to imiquimod at 5 μg/ml (1.7- and 56.3-fold, respectively, as determined by densitometric analysis), and the signal intensity decreased over time (Figure 1b). The TNFα signal showed a similar, albeit somewhat slower, upregulation (3.8-fold after 1 hour as determined by densitometric analysis; Figure 1c). On the protein level, we found clear induction of TNFα, IL-1β, IL-6, and IL-8 in cells incubated

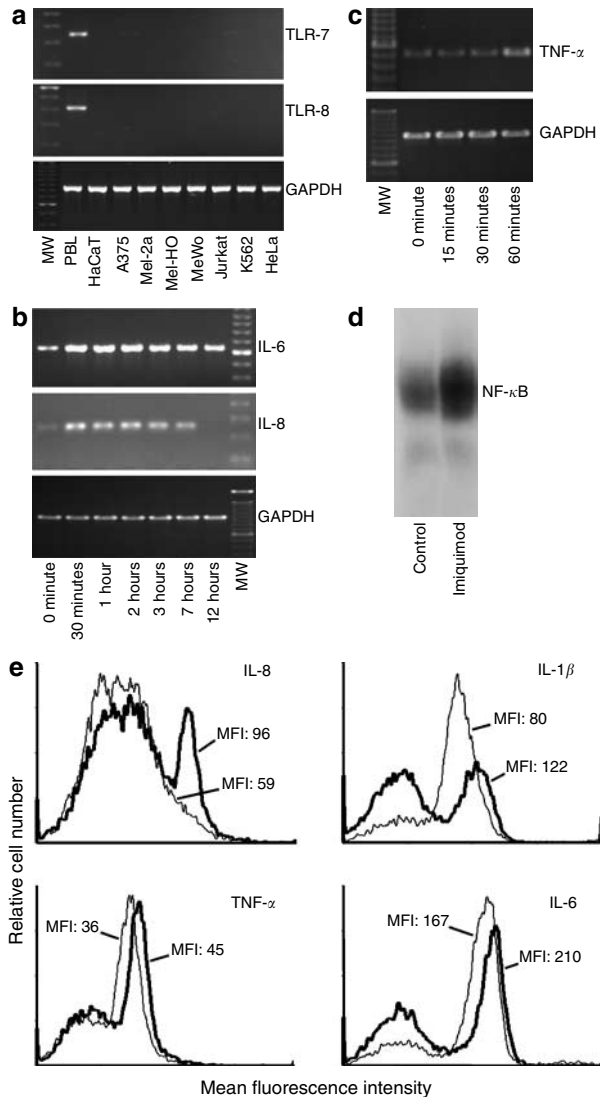

Figure 1. Imiquimod induces proinflammatory cytokines in TLR7- and TLR8-negative cells. (**a**) Expression of TLR7 (upper panel), TLR8 (middle panel), and GAPDH (loading control, lower panel) was assessed by RT-PCR in freshly isolated peripheral blood lymphocytes (positive control), the spontaneously immortalized human keratinocyte line HaCaT, the melanoma lines A375, Mel-2a, Mel-HO, and MeWo, the T lymphoblast line Jurkat, the erythroleukemia line K562, and HeLa tumor cells. (**b**) TLR7- and TLR8-negative HaCaT cells were incubated with imiquimod (5 μg/ml) for the indicated periods of time, and expression of IL-6 (upper panel), IL-8 (middle panel), and GAPDH (bottom panel) was assessed by RT-PCR. Both IL-6 and IL-8 are rapidly upregulated following exposure of the cells to imiquimod. (**c**) HaCaT cells were incubated with imiquimod (5 μg/ml) for the indicated time periods, and expression of TNFα (upper panel) and GAPDH (lower panel) was assessed by RT-PCR. (**d**) Activation of the transcription factor NF-κB was assessed in untreated HaCaT cells (left lane) and HaCaT cells treated with imiquimod (5 μg/ml for 1 hour) by an electrophoretic mobility shift assay using nuclear extracts as outlined in Materials and Methods. Please note imiquimod-induced activation of NF-κB. (**e**) HaCaT cells were incubated with imiquimod 5 μg/ml for 24 hours. Thereafter, cells were fixed, permeabilized, and the indicated intracellular cytokines were detected as outlined in Materials and Methods. In each panel, the thin curve represents untreated control cells and the bold-print curve represents imiquimod-treated cells. Please note that imiquimod induces expression of TNFα, IL-1β, IL-6, and IL-8 on the protein level.