Tumor Necrosis Factor-α and Interleukin-1 Antagonists Alleviate Inflammatory Skin Changes Associated with Epidermal Growth Factor Receptor Antibody Therapy in Mice

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Abstract
Cancer patients receiving epidermal growth factor receptor (EGFR) antibody therapy often experience an acneiform rash of uncertain etiology in skin regions rich in pilosebaceous units. Currently, this condition is treated symptomatically with very limited, often anecdotal success. Here, we show that a monoclonal antibody targeting murine EGFR, ME1, caused a neutrophil-rich hair follicle inflammation in mice, similar to that reported in patients. This effect was preceded by the appearance of lipid-filled hair follicle distensions adjacent to enlarged sebaceous glands. The cytokine tumor necrosis factor-α (TNFα), localized immunohistochemically to this affected region of the pilosebaceous unit, was specifically upregulated by ME1 in skin but not in other tissues examined. Moreover, skin inflammation was reduced by cotreatment with the TNFα signaling inhibitor, etanercept, indicating the involvement of TNFα in this inflammatory process. Interleukin-1, a cytokine that frequently acts in concert with TNFα, is also involved in this process given the efficacy of the interleukin-1 antagonist Kineret. Our results provide a mechanistic framework to develop evidence-based trials for EGFR antibody–induced skin rash in patients with cancer.

Introduction
Epidermal growth factor receptor (EGFR) is a membrane protein controlling proliferation and maturation of normal epithelial cells in skin, as well as cancer cells of epithelial origin (1, 2). EGFR targeting with the antibodies cetuximab and panitumumab is approved for the management of several cancer types. However, these agents also cause an inflammatory “acneiform” rash in skin regions rich in pilosebaceous units (3, 4). Skin inflammation develops days after the start of treatment with reddened elevations of the skin that may subsequently contain pus. Histologically, affected skin regions often present with neutrophil-rich inflammation in the superficial dermis and hair follicles (3, 5). Treatments for this rash are, at present, symptomatic due to uncertain etiology.

To aid in the development of mechanism-based treatment approaches, we have developed an animal model of EGFR antibody–induced skin inflammation using a rat monoclonal antibody targeting mouse EGFR, ME1 (6). Although the role of B cells and T cells in this inflammatory process has not been determined, a model was established in severe combined immunodeficient (SCID) mice that lack these cells, but retain neutrophils, to ensure that skin-inflammatory effects in our model were not due to an adaptive immune response to the foreign rat antibody ME1.

Materials and Methods
ME1 binding to mouse EGFR. ME1 (ImClone Systems) binding to recombinant human Fc-tagged mouse EGFR was determined by ELISA.

Animal models and dosing. Animal studies used female SCID mice (Charles River Laboratories), in accordance with current regulations and standards of the U.S. Department of Agriculture and the NIH. ME1, rat IgG (Equitech-Bio), and cetuximab (ImClone Systems) were administered i.p. at 40 mg/kg (Monday–Wednesday–Friday). Kineret (Ammgen; 150 mg/kg) and etanercept (Innogenex; 12.5 or 25 mg/kg) were administered i.p. daily.

Skin histology. Frozen (12 μm) or paraffinized (5 μm) skin sections were stained immunohistochemically with 3,3′-diaminobenzidine as chromagen, and/or with standard counterstaining methods.

Tumor necrosis factor-α, interleukin-1α, and interleukin-1β protein analysis. Cytokines were measured in skin homogenate and plasma with mouse-specific ELISA kits (R&D Systems).

Tumor necrosis factor-α, interleukin-1α, and interleukin-1β RNA analysis. Cytokine RNA expression was measured in perfused skin samples with Quantigene 2.0, 96-well plate assay kits (Panomics).

Statistical analyses. Skin cytokine levels, tissue mRNA levels, and the percentage of skin length with a neutrophil-rich inflammation from the study in which etanercept or Kineret treatment were started after 19 d of ME1 therapy were compared with a Wilcoxon rank sums test using JMP software from SAS. Tumor weights among the mice receiving anti-EGFR therapy, as well as plasma cytokine levels, were compared by one-way ANOVA followed by a Tukey-Kramer post hoc test. The percentage of skin length with a neutrophil-rich inflammation from the three pooled studies in which treatment was started 1 day prior to ME1 therapy was compared by standard least squares regression, with study and treatment as factors. For all analyses, n = number of mice and P < 0.05 was considered significant.

Results and Discussion
ME1 bound mouse EGFR (Supplementary Fig. S1) and caused a wavy hair phenotype in SCID mice (Fig. 1A), also reported for genetically engineered mice deficient in EGFR signaling (7).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Furthermore, ME1 prevented hair regrowth after shaving (Fig. 1B), similar to effects reported for a small molecule EGFR inhibitor (8).

Upon observing a yellowish crusting of the skin surface in shaved areas after 2 weeks of ME1 treatment, skin samples were harvested for histologic evaluation. ME1-treated skin showed the hallmarks of skin inflammation observed in patients treated with EGFR antibodies (4, 5), i.e., hair follicle plugging, pus-like neutrophil clusters outside the hair follicle canal, parakeratosis (retention of nuclei in stratum corneum cells), and ectatic (dilated) follicles (Fig. 1C). Neutrophil-rich inflammation with epidermal thickening was prominent in unshaved ME1-treated skin of the face and shoulders (Fig. 1D). In contrast, lower back skin inflammation was only significant when this region was shaved prior to the start of ME1 treatment, possibly related to the suggestion that patients may require additional stimuli (triggers) beyond EGFR inhibition to develop a skin rash (4).

Notably, ME1 treatment significantly increased the size of sebaceous glands in all skin regions (Fig. 1D; Supplementary Fig. S2), supporting a potential role for sebaceous glands in EGFR inhibitor–induced skin rash (3). Sebaceous glands, the overactivity of which is associated with acne vulgaris (9), predominately consist of mature oil-producing, EGFR-expressing sebocytes (ref. 10; Supplementary Fig. S3), that end their life cycle by disintegrating in a holocrine secretion process, releasing their contents into the hair follicle canal through a sebaceous duct.

In EGFR antibody–treated patients, skin rash begins ~1 to 3 weeks after the start of treatment (3, 5). In an effort to uncover the effects of ME1 preceding, and therefore, potentially contributing towards the development of skin inflammation, we next evaluated shoulder and lower back skin samples from unshaved mice after just 1 week of treatment (Supplementary Fig. S4). ME1 consistently increased the size of sebaceous glands, even prior to the onset of inflammation. Interestingly, enlarged sebaceous glands were observed to feed into oil-filled follicular distensions near sebaceous ducts in ME1-treated skin, but not in control skin (Fig. 2A). Antibody-mediated inhibition of EGFR may therefore

![Figure 1. ME1 causes effects consistent with EGFR inhibition. A, wavy hair after 7 d of ME1 treatment. B, hair regrowth within 21 d of shaving, prevented by ME1. C, H&E-stained facial skin after 14 d of ME1 treatment. E, ectatic follicles; K, keratin plug in a hair follicle; *, neutrophilic pus-like clusters; arrow, parakeratosis. D, sections of skin shaved 1 d prior to treatment start, or left unshaved, after 14 d of treatment (arrows, sebaceous glands). Anagen growth phase follicles penetrate the deep dermis of shaved skin in control mice only. Control, rat IgG; bars, 100 μm.](image-url)
Figure 2. Histologic effects of 7 d of ME1 treatment. A, Oil Red O–stained follicular distensions after ME1 treatment (arrows) in longitudinal or cross-sections of hair follicles of shoulder skin. B, neutrophil-rich hair follicle inflammation (arrows) observed in H&E-stained frozen section of shoulder skin after 7 d of ME1 treatment in an early phase just distal to the sebaceous gland (SG) and in a more advanced stage exiting the follicle. C, immunostaining with a nonspecific rabbit IgG in ME1-treated facial skin (top), compared with TNFα immunostaining after 7 d of the indicated treatment (middle and bottom). TNFα staining indicated in the sebaceous duct (arrow) and sebocytes near the site of holocrine secretion (arrowhead). Bar, 100 μm (A); bars, 50 μm (B and C). Control, saline (A and B); rat IgG (C).

Figure 3. ME1 treatment increases TNFα expression in skin. A, TNFα protein in skin homogenate during the first week of treatment (n = 6/group). B, TNFα mRNA in the indicated tissues after 1 wk of treatment (n = 4/group); *, P < 0.05 ME1 versus control. Control, rat IgG; bars, SE.
result in overactive sebaceous glands, in line with reports of reduced sebocyte maturation and oil production when EGFR is activated with ligands in vitro (11, 12). The general location of oil-filled follicular distensions induced by 1 week of ME1 treatment was similar to that of early observations of neutrophil-rich hair follicle inflammation (Fig. 2B).

The normal sebaceous gland of human and mouse skin is reported to express tumor necrosis factor-α (TNFα; ref. 13), an inflammatory cytokine that can cause neutrophil-rich skin inflammation (14). In control SCID mice, TNFα immunoreactivity was predominantly localized to the vicinity of the hair follicle sebaceous ducts and sebocytes (Fig. 2C). After 7 days of ME1 treatment, TNFα immunostaining remained focused on sebocytes and the adjacent distended follicular canals.

Beyond simply increasing the size of the skin region highest in TNFα, ME1 significantly increased TNFα protein levels in skin after 7 days ($P < 0.0008$), but not after 1 or 3 days of treatment (Fig. 3A). TNFα mRNA was similarly increased in ME1-treated skin, but not in other tissues (Fig. 3B; $P < 0.03$). This skin-focused tissue pattern of increased TNFα production is notably similar to that of EGFR antibody–associated toxicities in patients (3).

The above data support the hypothesis that targeting EGFR with ME1 increases sebocyte maturation, eventually resulting in sebaceous gland hypertrophy, follicular distension, and increased skin TNFα levels that could contribute towards skin inflammation. We therefore evaluated the effects of a concomitantly administered TNFα antagonist on GR-1–positive neutrophil-rich skin inflammation associated with ME1 treatment. To account for interstudy variability in ME1-induced skin inflammation potentially related to variation in triggers for rash that act with or apart from EGFR targeting, results were pooled from three studies evaluating therapy with etanercept, a fusion protein containing two extracellular domains from the human p75 TNFα receptor, joined to a single human IgG1 Fc region.

ME1 increased neuphilic skin inflammation in all three regions examined, especially the face and shoulder ($P < 0.0001$). Etanercept reduced this inflammation when daily treatment was started 1 day prior to the start of ME1 dosing (Fig. 4A; Supplementary Fig. S5; $P < 0.002$ for the face and shoulder, $P < 0.02$ for the lower back). The effects of ME1 were independent of the study used in the pooled analysis, although the effects of etanercept on facial skin inflammation varied between studies ($P < 0.0002$). This variation was due to a weaker effect on the ME1-aggravated facial rash associated with an eye irritation observed in one of the three studies (13) (Supplementary Fig. S5); IL-1α and IL-1β protein levels in skin after 16 d of therapy ($n = 5–6$ mice per group per skin region) were detectable and higher than control levels ($P < 0.05$ versus ME1 treatment). Rat IgG or rat IgG + human IgG, or human IgG (25 mg/kg), or Kineret started 1 d prior to anti-EGFR (ME1 + cetuximab) treatment did not significantly reduce skin inflammation (Supplementary Fig. S2).

TNFα frequently acts in concert with interleukin-1 (IL-1; refs. 15, 16). Although IL-1α and IL-1β protein levels in skin never exceeded the detection limits of our assays through 7 days of ME1 therapy (data not shown); IL-1α and IL-1β mRNA were detectable and showed trends for increase in various tissues (Supplementary Fig. S7), without the skin-focused pattern observed for TNFα. In


Figure 4. Effects of etanercept or Kineret cotreatment. A pooled analysis of individual mouse values for percentage of total skin sample length containing a GR-1–positive neutrophilic infiltration on the 16th to 19th d of ME1 treatment from three studies ($n = 5–6$ mice per group per skin region): etanercept (12.5 or 25 mg/kg) or Kineret started 1 d prior to ME1. Control, rat IgG or rat IgG + human IgG. B, plasma TNFα (top) and IL-1α (bottom; dashed lines, start of linear region of standard curve) after 16 d of therapy ($n = 6$ mice per group). Etanercept (12.5 mg/kg) or Kineret treatment started 1 d prior to ME1. Control, rat IgG + human IgG; *, $P < 0.05$ versus ME1 + human IgG. C, GEO human colorectal cancer tumor mass after 17 d of treatment with etanercept, human IgG (25 mg/kg), or Kineret started 1 d prior to anti-EGFR (ME1 + cetuximab) or control (rat IgG + human IgG) treatment ($n = 11–12$ mice per group); *, $P < 0.05$ versus anti-EGFR; bars, SE.
line with an important role for IL-1 signaling in this model, recombinant IL-1 receptor antagonist, Kineret, reduced inflammatory skin rash in ME1-treated mice, independent of the study (Fig. 4A; Supplementary Fig. S5; \( P < 0.0007 \) for the face and shoulder, \( P = 0.06 \) for the lower back). Importantly, whereas neutrophilic infiltrates of the skin were significantly reduced with either etanercept or Kineret, neither treatment prevented the occurrence of wavy hair, sebaceous gland enlargement, or hair follicle distension (Supplementary Fig. S8). TNFα and IL-1 therefore act downstream of these effects of ME1 to predispose mice to hair follicle inflammation.

IL-1 might act upstream of TNFα in some instances (17). In the present model however, in 2 of 3 studies, Kineret did not significantly reduce an ME1 induced increase in plasma TNFα (Fig. 4B). However, in a third study, Kineret lowered plasma TNFα (data not shown), indicating that further evaluation is necessary. In contrast, etanercept blocked an increase in plasma IL-1α in three of three studies (Fig. 4B), supporting the possibility that TNFα acts upstream of IL-1 signaling in mediating the inflammatory effects of ME1.

From day 19 to day 36 of ME1 treatment, skin inflammation tended to decrease in severity in the face, while spreading caudally to the lower back (Supplementary Fig. S9). When the start of Kineret and etanercept treatments were delayed to day 19, positive effects were again observed, although they did not reach statistical significance (\( P > 0.06 \)). ME1 treatment cessation (switch to Rat IgG on day 19) significantly reduced rash on the face and shoulder, and minimized its spread to the lower back (\( P < 0.02 \)).

TNFα has the potential to accelerate (18, 19) or impede (20) cancer progression. Thus, systemic administration of TNFα inhibitors, while alleviating the skin rash during EGFR antibody therapy, may also affect anticancer efficacy. To begin to explore this important consideration, we evaluated the effects of etanercept and Kineret on the efficacy of combined antibody-mediated inhibition of mouse (ME1) and human (cetuximab) EGFR in a SCID mouse subcutaneous xenograft tumor model, established with GEO human colorectal cancer cells. Kineret had no significant effect on the antitumor effect of anti-EGFR therapy, but a moderate loss of efficacy was observed with etanercept (Fig. 4C). Although additional testing is still necessary to determine the general applicability of this finding in other tumors, the results do suggest that TNFα may augment the antitumor effects of EGFR antibody–targeted therapy.

In conclusion, current treatment guidelines for EGFR antibody–induced skin rash have not been developed with the benefits of an understanding of the underlying mechanism. Here, we report on a mechanism uncovered in an animal model of this condition, offering a rationale that can be tested further in translational studies evaluating the importance of EGFR antibody–induced TNFα and IL-1 signaling in predisposing patients to skin inflammation.

Disclosure of Potential Conflicts of Interest

Acknowledgments
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