Role of epidermal growth factor and its receptor in chemotherapy-induced intestinal injury

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Huang, Frederick S., Christopher J. Kemp, Jodi L. Williams, Christopher R. Erwin, and Brad W. Warner. Role of epidermal growth factor and its receptor in chemotherapy-induced intestinal injury. Am J Physiol Gastrointest Liver Physiol 282: G432–G442, 2002.—Several growth factors are trophic for the gastrointestinal tract and able to reduce the degree of intestinal damage caused by cytotoxic agents. However, studies of epidermal growth factor (EGF) for chemotherapy-induced intestinal injury are conflicting. The development of a transgenic mouse that specifically overexpresses EGF in the small intestine provided a unique opportunity to assess the contribution of EGF in mucositis. After a course of fluorouracil, transgenic mice fared no better than control mice. Weight recovery was inferior, and mucosal architecture was not preserved. Apoptosis was not decreased and proliferation was not increased in the crypts. To corroborate the findings in transgenic mice, ICR mice were treated with exogenous EGF after receiving fluorouracil. Despite ileal upregulation of native and activated EGF receptor, the mice were not protected from intestinal damage. No benefits were observed with different EGF doses or schedules or routes of EGF administration. Finally, mucositis was induced in mutant mice with specific defects of the EGF signaling axis. Compared with control mice, clinical and histological parameters of intestinal injury after fluorouracil were no different in waved-2 mice, which have functionally diminished EGF receptors, or waved-1 mice, which lack transforming growth factor-α, another major ligand for the EGF receptor. These findings do not support a critical role for EGF or its receptor in chemotherapy-induced intestinal injury.

intestinal mucosa; fluorouracil; enteritis; transgenic mouse; transforming growth factor-α

CHEMOTHERAPY-INDUCED INTESTINAL injury, commonly referred to as mucositis, is a major cause of morbidity and mortality during therapy for cancer. This problem has become more clinically relevant as oncologists have intensified chemotherapeutic regimens in an effort to improve long-term survival (1). Neutropenic enterocolitis represents the most severe manifestation of this complication and has an incidence and mortality as high as 32% and 45%, respectively (16, 34).

Mucositis is characterized by the loss of intestinal mucosal integrity that results from the widespread apoptosis and death of regenerative stem cells secondary to chemotherapy (29). The ensuing damage includes perturbed brush-border hydrolase activity (28), reduced mucosal DNA, RNA, and protein content (25), blunted villus heights and crypt depths (7, 10), and decreased proliferation (7, 10, 32) and increased apoptosis of crypt cells (27). Additionally, there is an increase in mucosal permeability (15, 25, 32), an important predisposing factor to the translocation of bacteria from the intestinal lumen to the bloodstream and distant organs (2, 3). These cellular changes ultimately result in a clinical syndrome of diarrhea, intestinal blood loss, dehydration, impaired nutrition, weight loss, and disseminated infections (7, 10, 15).

Several growth factors have been studied for their potential to attenuate or even nullify cytotoxic intestinal injury (5, 14, 30, 36, 38). Interleukin-11 (IL-11) and keratinocyte growth factor (KGF) have been the recent focus of investigation, and the results of initial studies are promising. They appear to confer significant protection on the gastrointestinal tract from cytoablative therapy (7, 10, 26, 27). These findings have been replicated in other models of intestinal injury (6, 17, 20, 31, 33, 40). Epidermal growth factor (EGF) also exerts beneficial effects in these models (4, 18, 19, 21, 39), but its role in mucositis has not been studied extensively and its efficacy remains unclear (13, 28, 32, 35).

Erwin and colleagues (9) have created a transgenic mouse that constitutively overexpresses EGF. By joining the promoter for rat intestinal fatty acid-binding protein and the coding sequence for murine EGF, Erwin et al. (9) designed a construct that targeted overexpression exclusively to the villus enterocytes of the jejunum and ileum. Intestinal EGF levels were ~60% higher in the transgenic mice compared with nontransgenic littermates. Furthermore, these levels were physiologically significant and provided a clinical advantage. After a small bowel resection, the DNA and protein content and villus heights and crypt depths of the remaining intestine were increased in the trans-
genic mice, findings that are consistent with enhanced adaptation. This study exploited the intestine-specific overexpression of EGF in these transgenic mice to evaluate the effects of this growth factor in chemotherapy-induced intestinal injury. In separate experiments, fluorouracil-treated ICR mice were administered either subcutaneous EGF or albumin, and the degree of intestinal injury was measured. The expression of EGF receptor in the intestine of these animals after fluorouracil also was investigated. In addition, mucositis was induced in two mutant murine strains with different defects of the EGF signaling axis. The significance of the EGF receptor was tested in waved-2 mice, which possess an abnormal EGF receptor with markedly decreased activity (22). Waved-1 mice, which are deficient in transforming growth factor-α (TGF-α), were used to assess the contribution of another major ligand for the EGF receptor (23, 24).

METHODS

Animals. The protocol for this study was approved by the Children’s Hospital Research Foundation Institutional Animal Care and Use Committee. Mice homozygous for a transgene that results in the targeted overexpression of murine EGF in the small intestine were utilized. The genotype of these transgenic mice was confirmed by PCR of DNA isolated from ear clips as previously described (9). FVB mice (Taconic, Germantown, NY) served as background strain controls. ICR mice (Harlan, Indianapolis, IN) were used in experiments involving exogenous EGF. Studies with the waved-2 and waved-1 strains (Jackson Laboratory, Bar Harbor, ME) included C57BL/6 mice (Jackson Laboratory) as controls. All mice were male, 25–30 g in weight, housed in groups of four at 21°C on 12:12-h light-dark cycles, fed standard rodent chow ad libitum, and allowed to acclimate to their environment for at least 4 days before experimentation.

Experimental designs. After confirming the ideal overexpression of the EGF construct in the transgenic mouse by RT-PCR, we gave the transgenic and FVB strains either fluorouracil or saline for 4 days. In one set of experiments, the mice were weighed and observed for toxicity during and after the chemotherapy or vehicle for 9 days. In another set of experiments, the mice were killed 2 days after completing the course of fluorouracil or saline. The ileum was then harvested and prepared for the histological studies.

The ICR mice were given fluorouracil as a single dose and then treated with either exogenous EGF or albumin daily until the end of the experiment. In the first set of experiments, weights were recorded and toxicity was monitored for 14 days. In the second set of experiments, the ileum was harvested 2, 4, 8, and 12 days after fluorouracil administration and then prepared for the histological studies and Western blot analysis.

The waved-2, waved-1, and C57BL/6 mice were administered a single dose of fluorouracil or saline, and their weights and clinical status were followed for 20 days. Ileum was obtained from another cohort of mice 4 and 20 days after fluorouracil or saline for the histological studies.

We chose the experimental days on which more detailed histological analyses were performed to coincide with the intestinal injury and repair phases after fluorouracil administration. These phases were determined from the weight and survival curves, which vary with murine strain, and the different time points for each of the experiments reflect this biological variability.

Fluorouracil and EGF administration. Fluorouracil (Sigma Chemical, St. Louis, MO) was diluted in saline adjusted to pH 10 with sodium hydroxide. A total of 200 mg/kg fluorouracil given as four consecutive daily doses of 50 mg/kg was administered intraperitoneally to the transgenic and FVB mice. The ICR mice received a single dose of 200 mg/kg fluorouracil. The waved-2, waved-1, and C57BL/6 mice received a single dose of 225 mg/kg fluorouracil. These fluorouracil dosages were chosen to effect a similar degree of intestinal damage and clinical toxicity among the various strains of mice and were determined in separate dose-toxicity experiments based, in part, on fluorouracil dosages established in other studies (7, 10). An equal volume of the basic saline vehicle was given to the mice that were not assigned to receive chemotherapy.

EGF (Chiron, Emeryville, CA) was diluted in saline, and a dose of 100 μg/kg was injected subcutaneously daily until the end of the experiment. An equal volume of 1% BSA (Life Technologies, Grand Island, NY) was given to the mice that were not randomized to receive EGF. All injections were performed under brief inhaled isoflurane anesthesia.

Tissue harvest. All mice were killed with an intramuscular injection of a ketamine-xylazine-acepromazine cocktail (4:1:1 proportion) followed by cervical dislocation. A 6-cm section of distal ileum immediately adjacent to the ileocecal valve was excised. The distal 1-cm portion was placed in 10% buffered formalin for the histological studies, and the remainder was frozen at −80°C for RT-PCR in the experiments with the transgenic and FVB mice or Western blot analysis in the experiments with ICR mice. A 5-cm segment of jejunum also was obtained from the transgenic and FVB mice.

RT-PCR. RNA was isolated from the intestinal samples with TRIzol (Life Technologies). After the RNA concentration of each sample was determined spectrophotometrically, 20 μg RNA from each sample were pooled into the appropriate experimental group.

cDNA was produced from 5 μg RNA from each experimental group using SuperScript II. RT (Life Technologies). A PCR for EGF construct cDNA was performed with the oligonucleotide primer pair 5′-ACA TAG ATG GAA TGG GCA CAG G-3′ and 5′-CCT CTA GAA ATG TGG TAT GGC TG-3′ (Life Technologies). Equal loading of 2 μg of cDNA from each experimental group was confirmed by probing simultaneously for 18S cDNA with an oligonucleotide primer pair from a commercial kit (Ambion, Austin, TX). The resulting EGF construct and 18S products were 286 and 488 bp, respectively. Thirty cycles were required for sufficient resolution by electrophoresis on a 1% agarose gel.

Rat intestinal fatty-acid binding protein plasmids containing the EGF construct served as a positive control. Water was substituted for cDNA as a negative control.

Histology and morphometry. After fixation for 24 h and routine processing, samples of ileum were oriented and embedded in paraffin to provide cut sections parallel with the longitudinal axis of the bowel. Five-micrometer sections were mounted on electrostatically treated slides (Fisher Scientific, Pittsburgh, PA) and stained with hematoxylin and eosin or used for immunohistochemical studies.

Measurements of the villus heights and crypt depths were performed on the hematoxylin and eosin-stained sections using a light microscope with a video camera (Dage-MTI, Michigan City, IN) attached to an image capture card (Integra Technologies, Indianapolis, IN) in a standard desktop computer with image analysis software (ImageTool, University of Texas Health Science Center, San Antonio, TX). At
least 20 intact villi and crypts were counted and averaged for each sample.

**Apoptosis.** The hematoxylin and eosin-stained sections also were used to assess crypt apoptosis. The ratio of the number of crypt cells with the morphological features of apoptosis (nuclear condensation and segmentation, cell shrinkage, and apoptotic bodies) to the total number of cells in a crypt was determined for at least 20 crypts for each sample. The values were averaged to produce the mean percentage of crypt apoptosis.

**Proliferation.** Proliferation of the crypt cells was detected with a monoclonal antibody for proliferating cell nuclear antigen (PCNA) using a commercial kit (Zymed Laboratories, South San Francisco, CA). Formalin-fixed, paraffin-embedded ileal sections were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was quenched by incubating the sections in a 3% hydrogen peroxide and methanol solution, and nonspecific antigens were neutralized by treating the sections with the included blocking solution for 30 min. Detection of the biotinylated PCNA antibody was accomplished with a streptavidin-horseradish peroxidase conjugate as the signal generator and diaminobenzidine as the chromogen. The sections were counterstained with 1% methyl green, dehydrated in butanol, and cleared in xylene. The ratio of the number of crypt cells staining positively for PCNA to the total number of cells in a crypt was determined for at least 20 crypts for each sample, and the values were averaged to produce the mean percentage of crypt proliferation.

**Western blot analysis.** The ileal samples were homogenized and then purified by ultracentrifugation at 50,000 g for 1 h. After determining the protein concentration of each sample with a modified Lowry assay (Pierce, Rockford, IL), 250 μg of protein from each sample were pooled into the appropriate experimental group. The EGF receptor was immunoprecipitated from 500 μg of protein from each experimental group with rabbit anti-EGF receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 2 μg/ml and 20 μl of protein A–agarose conjugate (Santa Cruz Biotechnology) according to the manufacturer’s suggested protocol. With the use of a 4–20% polyacrylamide gel (Invitrogen, Carlsbad, CA), the immunoprecipitates were separated electrophoretically and then transferred overnight onto a polyvinylidene difluoride membrane (Osmonics Laboratory Products, Minnetonka, MN).

After blocking nonspecific antigens with 1% BSA for 1 h, detection of activated EGF receptor was accomplished by incubating the membrane with a mouse anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY) diluted to 1 μg/ml for 1 h followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Transduction Laboratories) diluted to 0.1 μg/ml for 1 h. The antibodies were revealed with chemiluminescence (NEN Life Science Products, Boston, MA) and exposure onto autoradiography film (Eastman Kodak, Rochester, NY). After the antibodies were removed with a stripping buffer (Pierce), the same membrane was reprobed for EGF receptor in a similar manner. For the detection of native EGF receptor, a rabbit anti-EGF receptor antibody (Santa Cruz Biotechnology) diluted to 0.4 μg/ml and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Calbiochem-Novabiochem, San Diego, CA) diluted to 0.02 μg/ml were utilized. Nonspecific antigens were blocked with 5% nonfat milk.

Lysates from A431 cells and EGF-stimulated A431 cells (Transduction Laboratories) were used as positive controls.

**Statistical analysis.** Results are expressed as means ± SE. Analyses of the results were performed with statistical software (SigmaStat, SPSS, Chicago, IL). Statistical differences were determined with an unpaired t-test or a two-way ANOVA, followed by pairwise comparisons utilizing a Student-Newman-Keuls test. P < 0.05 was considered significant.

**RESULTS**

To validate the primary characteristic of these transgenic mice, EGF construct expression in the jejunum and distal ileum was assessed by RT-PCR. Consistent with the original description of these animals, EGF construct was present in the jejunum of the transgenic mice (Fig. 1). Of particular note, EGF construct also was observed in the distal ileum. As expected, no EGF construct was evident in the jejunum or distal ileum of the FVB mice. The expression of EGF construct in the distal ileum of the transgenic mice, albeit somewhat less than that of the jejunum, justifies the use of this intestinal segment and this transgenic murine strain in these experiments.

The transgenic and FVB mice developed diarrhea, dehydration, anorexia, and weight loss upon receiving 200 mg/kg of fluorouracil. Despite these clinical findings, the survival rate was 100%. The pattern of initial weight loss was similar for the transgenic and FVB mice (Fig. 2). Weight loss started on the 4th day of experiments and reached a maximum of ~10% by the 6th day. However, the pattern of subsequent weight recovery differed significantly. The FVB mice rapidly returned to their baseline weight, fully recovering by the 8th day. In contrast, the transgenic mice regained their weight more slowly and incompletely, ultimately recovering only 50% of their weight loss. The transgenic and FVB mice that were administered saline were asymptomatic and did not have weight loss.

In separate experiments, transgenic and FVB mice were given fluorouracil as described, but they were killed and their ileum harvested on the 6th experimental day, coincident with the time of maximal weight loss and before the start of weight recovery. Histological manifestations of chemotherapy-induced damage were evident in the tissue sections from the FVB and transgenic mice (Fig. 3, C and D). Distortion of the mucosal architecture, particularly of the villi, was
readily apparent. Blunting of the villi and sloughing of the enterocytes into the intestinal lumen were present. These findings were absent in the FVB and transgenic mice that received saline (Fig. 3, A and B).

Measurements of the villus heights and crypt depths substantiated the histological observations. The villus heights of the fluorouracil-treated mice were significantly shorter compared with mice given saline, demonstrating the injurious effects of chemotherapy on the ileum (Fig. 4A). In contrast, the crypt depths were greater in the mice that received fluorouracil, reflecting the intestinal response that follows injury and precedes repair (Fig. 4B). However, neither the villus heights nor the crypt depths were significantly different after the administration of fluorouracil in the transgenic mice compared with FVB mice. These results were confirmed with crypt depth-to-villus height ratios, which afford greater precision (Fig. 4C).

Because chemotherapeutic agents preferentially target rapidly dividing cells, including those of the intestinal crypt, the extent of apoptosis in the crypts was evaluated as another parameter of intestinal damage. A fivefold increase in crypt apoptosis was induced in the mice treated with fluorouracil, but apoptosis was not decreased in the transgenic mice compared with FVB mice (Table 1). As observed with the crypt depths, significant increases in intestinal stem cell proliferation were seen in both strains of mice after receiving fluorouracil. However, crypt proliferation was not augmented in the transgenic mice compared with FVB mice (Table 1). Similar results were obtained when the magnitude of crypt apoptosis and proliferation was expressed as an absolute value (number of apoptotic or PCNA-positive cells per crypt).

To corroborate the negative findings in the transgenic mice, we performed experiments with exogenously administered EGF in ICR mice given fluorouracil. Compared with mice that received albumin, no improvements in the survival rate were seen with EGF (Fig. 5A). Although no differences in weight were observed initially, the EGF-treated mice developed an advantage in weight 6 to 10 days after fluorouracil administration (Fig. 5B). Utilizing another cohort of
mice, we evaluated histological parameters during both the injury and repair phases subsequent to fluorouracil administration. With the singular exception of the villus heights 4 days after fluorouracil administration, EGF treatment did not result in preserved villus heights (Fig. 6A), attenuated crypt apoptosis (Fig. 6B), or enhanced crypt proliferation (Fig. 6C) at any time point after chemotherapy. The increased villus heights of the mice administered EGF were not sustained beyond the 4th experimental day, and no other significant changes were demonstrated during the repair phase.

The ileum of these mice also was assayed for EGF receptor expression (Fig. 7). There was not a significant upregulation of native EGF receptor or its tyrosine-phosphorylated, activated form at any time point in the mice given albumin after fluorouracil compared with unperturbed mice that received no chemotherapy. However, as expected, the mice that received exogenous EGF after fluorouracil administration demonstrated increased ileal expression of activated EGF receptor compared with albumin-treated mice. Although there are two bands that approximate 170 kDa on the Western blot, the band that represents the native and activated EGF receptor is the upper one, which aligns with the bands of the positive controls. The lower one is most likely a truncated, variant form of the EGF receptor. The diminished intensity of the native EGF receptor bands for the positive controls is the result of the stripping buffer. Because the cell lysates are diluted to avoid an excessively intense and uninterpretable band, partial antigen loss from the action of the stripping buffer is more evident.

To exclude the possibility that a different EGF dose or schedule or route of EGF administration would be

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Table 1. Crypt apoptosis and proliferation after fluorouracil in transgenic mice

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Apoptosis, % of cells</th>
<th>Proliferation, % of cells</th>
</tr>
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<tbody>
<tr>
<td>FVB saline</td>
<td>0.7 ± 0.2</td>
<td>34.2 ± 2.6</td>
</tr>
<tr>
<td>FVB fluorouracil</td>
<td>3.6 ± 0.5*</td>
<td>71.6 ± 4.3*</td>
</tr>
<tr>
<td>Transgenic saline</td>
<td>0.8 ± 0.2</td>
<td>28.9 ± 4.4</td>
</tr>
<tr>
<td>Transgenic fluorouracil</td>
<td>4.7 ± 0.7*</td>
<td>66.1 ± 2.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Crypt apoptosis and proliferation in transgenic (n = 7) and FVB mice (n = 4) 2 days after administration of intraperitoneal fluorouracil (200 mg/kg) and transgenic (n = 4) and FVB mice (n = 4) 2 days after intraperitoneal saline. Values in % represent % of apoptotic or proliferating cell nuclear antigen (PCNA)-positive cells per crypt. No. of cells refers to absolute no. of apoptotic or PCNA-positive cells per crypt. *P < 0.05 for transgenic or FVB mice after fluorouracil vs. same mice after saline.

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Fig. 4. Villus height (A), crypt depth (B), and ratio of crypt depth (CD) to villus height (VH; C) of transgenic (n = 7) and FVB mice (n = 4) 2 days after 200 mg/kg of intraperitoneal fluorouracil and transgenic (n = 4) and FVB mice (n = 4) 2 days after intraperitoneal saline. Values are means ± SE. *P < 0.05 for transgenic or FVB mice after fluorouracil vs. same mice after saline (by 2-way ANOVA and Student-Newman-Keuls test).
more beneficial, mucositis was induced in ICR mice with 500 mg/kg of fluorouracil and the effect of these variables was investigated. No EGF dose (50–10,000 µg·kg⁻¹·day⁻¹) or schedule (before, during, and after fluorouracil) or route of EGF administration (subcutaneous and orogastric) was found to increase the survival rate or decrease the amount of weight loss (data not shown).

As another means of assessing the significance of the EGF receptor, we studied the effect of a functionally attenuated EGF receptor during and after mucositis in waved-2 mice. Except for a period of time from the 8th to the 13th experimental day during which the

Fig. 5. Survival rates (A) and weight curves (B) of EGF- (n = 8) and albumin-treated ICR mice (n = 8) after 200 mg/kg of intraperitoneal fluorouracil. Values are means ± SE. *P < 0.05 for EGF- vs. albumin-treated mice (by 2-way ANOVA and Student-Newman-Keuls test).

Fig. 6. Villus height (A), crypt apoptosis (B), and crypt proliferation (C) of EGF- (n = 8) and albumin-treated ICR mice (n = 8) 2, 4, 8, and 12 days after 200 mg/kg of intraperitoneal fluorouracil. Values are means ± SE. *P < 0.05 for EGF- vs. albumin-treated mice (by unpaired t-test).
waved-2 mice had significantly greater weights than the C57BL/6 mice, both murine strains had nearly identical clinical courses after receiving fluorouracil, with similar survival rates (Fig. 8A) and patterns of weight loss (Fig. 8B). In separate experiments, the ileum of these mice was examined during and after the intestinal injury resulting from fluorouracil. The villus heights of the waved-2 mice were not significantly different at any time point compared with C57BL/6 mice (Fig. 8C). The mice that received saline did not exhibit any clinical toxicity or histological damage.

Similar responses were seen when the contribution of another major ligand for the EGF receptor was evaluated in waved-1 mice. No differences in survival rates (Fig. 9A), weight loss (Fig. 9B), or villus heights (Fig. 9C) were noted between the waved-1 and C57BL/6 mice after fluorouracil.

DISCUSSION

To address the questions that have arisen regarding the efficacy of EGF in mucositis, this study employed different but complementary experimental approaches to establish that this growth factor has a limited role in chemotherapy-induced intestinal injury. First, the experimental design of all prior investigations (13, 28, 32, 35) of EGF and mucositis has been based upon and restricted to the exogenous administration of the growth factor. In addition to extensive tests of exogenous EGF, this study utilized transgenic mice that overexpress EGF in the small intestine and mutant mice that lack TGF-β to demonstrate that neither EGF nor different ligands for the EGF receptor contribute significantly to the prevention or repair of intestinal damage caused by chemotherapy. Second, the status of the EGF receptor in mucositis has not been assessed previously. This study revealed that neither the activation of this receptor by exogenous EGF nor the attenuation of this receptor in mutant mice greatly influences the histological or clinical manifestations of mucositis.

Other studies have yielded conflicting conclusions regarding the benefits of EGF in chemotherapy-induced intestinal injury. Focusing on weight loss, [14C]xylose uptake, and crypt survival, Robinson and co-workers (32) could not demonstrate a consistent advantage with intraperitoneal or orogastric EGF in mice with…
mucositis secondary to melphalan. Using biochemical parameters, Petschow and associates (28) concluded differently and showed that EGF accelerates the recovery of intestinal brush-border hydrolase activities after methotrexate in rats. Animals that received EGF in their liquid diet had more normal levels of leucine aminopeptidase, maltase, and sucrase activity in the middle portion of their small intestine 6 days after chemotherapy. Interestingly, higher doses of EGF resulted in blunted levels of disaccharidase activity in the same section of intestine, revealing a possible dose limit. Hirano et al. (13) also found that EGF facilitates intestinal mucosal recovery after methotrexate in rats. The activity of ornithine decarboxylase, an enzyme that participates in the repair of damaged rat intestinal mucosa, occurred earlier and was greater in animals given intraperitoneal injections of EGF. Sonis and co-workers (35) reported that EGF actually exacerbates oral mucositis in hamsters. When a continuous infusion of EGF to the oral cavity was initiated after the induction of mucositis by intraperitoneal fluorouracil and oral trauma, severity scores based upon the gross appearance of the buccal mucosa were significantly higher.

In contrast to those of EGF, studies of other growth factors have been uniformly positive. Du and coworkers (7) demonstrated that IL-11 can stimulate recovery of the intestinal mucosa, reduce the dissemination of enteric bacteria to distant organs, and increase survival in mice after combined chemotherapy and radiotherapy. In another series of experiments, Orazi et al. (27) showed that the intestinal regeneration mediated by IL-11 occurs not only through increased proliferation but also decreased apoptosis of the crypt cells. Opal et al. (26) found that the combination of IL-11 and granulocyte colony-stimulating factor provides additive protection against infectious complications related to intestinal damage after chemotherapy. Farrell and colleagues (10) discovered that KGF also reduces the morbidity and mortality of mucositis by stimulating crypt cell proliferation and enhancing crypt survival. Similar results have been reported with growth hormone (30), insulin-like growth factor I (14), neurotensin (38), bombesin (5), and glucagon-like peptide 2 (36).

The discordant results obtained with EGF and the preponderance of positive data generated by other growth factors have been difficult to reconcile, thus prompting the experiments in this study.

The use of a transgenic mouse to evaluate the efficacy of a growth factor in chemotherapy-induced intestinal injury is novel. The primary advantage of this experimental approach is the minimization of confounding factors that are introduced with the exogenous delivery of any substance, particularly the stress that is associated with orogastric, subcutaneous, or
intraperitoneal administration in animals (28). The organ-specific restriction of the growth factor of interest also reduces any systemic effects that may result when the exogenous form is given enterally or parenterally (9). The EGF-overexpressing transgenic mice utilized in this study possessed both of these advantages. The results obtained in these mice after fluorouracil administration strongly suggest that EGF does not exert a beneficial effect in chemotherapy-induced intestinal injury.

The lack of protection from mucositis in the transgenic mice may be related to the constitutive pattern of EGF expression by the enterocytes on the villi. The continuous production of growth factor effectively provides these enterocytes with EGF stimulation before, during, and after their exposure to fluorouracil. These constitutively activated enterocytes may have been made more susceptible to the effects of the fluorouracil, nullifying any potential benefit of EGF. A sufficiently significant loss of enterocytes also would have rendered the continued presence of EGF after the clearance of fluorouracil ineffective. However, this possibility is difficult to support given the negative findings in the ICR mice, which did not receive EGF until the dose of fluorouracil was delivered. Furthermore, no improvements were seen in these mice when various changes were made to the timing of exogenous EGF administration with fluorouracil administration.

A suboptimal intestinal concentration of EGF is another possible explanation for the inability of the transgenic mice to prevent or minimize the chemotherapy-induced intestinal damage. However, when Erwin and co-workers (9) initially characterized this strain, they found that the EGF level in intestinal homogenates was ~60% higher than that of the nontransgenic mice. When the transgenic mice were subjected to a small bowel resection, this EGF level resulted in the augmentation of the postoperative adaptive response in the remaining ileum. Therefore, independent of the amount of EGF that these animals actually overexpress in their small intestine, the effects of the EGF transgene translate into measurable physiological changes. In fact, the adaptive advantage afforded by this transgene in a model of small bowel resection only highlights the significance of the lack of protection that it provides from mucositis. Furthermore, as with the various schedules of EGF administration, a wide range of EGF doses were tested in the ICR mice, and no benefit was observed.

Although significantly increased weights were noted in the ICR mice that received exogenous EGF 6 to 10 days after the fluorouracil, they were not associated with concurrent benefits at a histological level, such as augmented villus heights, attenuated crypt apoptosis, or enhanced crypt proliferation. Therefore, it is difficult to attribute the weight improvement to intestinal protection by EGF. An isolated increase in the villus heights of these mice was observed 4 days after fluorouracil, but the clinical significance of this singular histological finding is diminished by the lack of a concomitant advantage in weight or survival at the same time point. Also, there was no decrease in crypt apoptosis or increase in crypt proliferation preceding the increased villus heights, which would be expected as part of the biological sequence of events. Additionally, this augmentation was unsustainable, with the difference in villus heights between the EGF-treated and albumin-treated mice being insignificant by the 8th and 12th experimental days. When considered as a whole, the weight and villus height data are not sufficient pieces of evidence for consistent and durable protection from mucositis with exogenous EGF. Furthermore, no advantage was seen at any time point for the other clinical and histological parameters of intestinal injury, including survival rate, crypt apoptosis, and crypt proliferation. These lines of analysis strongly suggest that the isolated weight and villus height findings represent a statistical aberration and not a relevant physiological event.

The Western blot analysis revealed that fluorouracil-induced intestinal damage does not increase the native or activated form of EGF receptor in the ileum, suggesting that this growth factor and its receptor have a minimal role in this type of gastrointestinal injury. Additionally, despite the expected ileal upregulation of activated EGF receptor by exogenous EGF, no histological or clinical protection from mucositis was observed. This apparent disconnection between receptor activation and mucosal repair may account for the inability of EGF, endogenous or exogenous, to attenuate chemotherapy-induced intestinal injury. The increased activation of EGF receptor after the administration of exogenous EGF also confirms the functional integrity of the receptor and diminishes the possibility of a specific effect of fluorouracil on the receptor as a mechanism for the negative histological and clinical findings.

The absence of increased toxicity and damage after fluorouracil in waved-2 mice, which do not have fully functional EGF receptors, is additional evidence that this growth factor receptor is not essential in mucositis. Although significantly greater weight differences were seen in the waved-2 mice 8 to 13 days after fluorouracil, this finding is not consistent with the hypothesis that the absence of an intact EGF receptor is clinically detrimental in chemotherapy-induced intestinal injury, which would predict that waved-2 mice should have greater weight loss after fluorouracil than C57BL/6 mice. Helmrath and associates (12) found that the degree of adaptation of the remaining ileum after a small bowel resection is reduced in waved-2 mice. Utilizing another model of intestinal injury, Egger and colleagues (8) reported that waved-2 mice are more susceptible to colitis caused by dextran sodium sulfate. These two studies (8, 12) demonstrate the increased sensitivity of this mutant murine strain to intestinal insults that are known to respond to EGF and underscore the limited role of the EGF receptor in damage secondary to chemotherapy (4, 21).

The lack of increased histological injury or clinical morbidity in fluorouracil-treated waved-1 mice reveals that TGF-α is not required for protection from mucosi-
tis. These results not only diminish the possibility that other ligands for the EGF receptor may be more relevant in mucositis, but also further deemphasize the importance of the EGF signaling axis in chemotherapy-induced intestinal injury.

Troyer and co-workers (37) have characterized the mucosa of the gastrointestinal tract in mice that do not express amphiregulin, EGF, or TGF-α. Three of the six ligands capable of activating the EGF receptor. These triple null (aaeett) mice develop spontaneous duodenal ulcers and possess transient ileal fragility, but are not more susceptible to colitis secondary to dextran sulfate sodium. Troyer et al. (37) postulate that aaeett mice may be more resistant to this type of injury than waved-2 mice (8) because of the contribution of betacellulin, epiregulin, and heparin-binding EGF, the other three ligands for the EGF receptor. Waved-2 mice express a defective receptor and therefore are insensitive to all of the ligands in the EGF family. In this study, redundancy along the EGF signaling axis may account for the absence of toxicity that was observed in the waved-1 mice after chemotherapy. If such a redundancy is operative in the biology of mucositis, even aaeett mice may not exhibit significant gastrointestinal damage secondary to chemotherapy. However, the contribution of different ligands cannot explain the lack of injury seen in the waved-2 mice that received fluorouracil. These findings suggest that it is unlikely any of the ligands in the EGF family can alter the course of chemotherapy-induced intestinal injury.

Various chemotherapeutic agents, including melphalan (32), methotrexate (13, 28), and fluorouracil (35), have been utilized in previous studies of EGF for mucositis. The variable results of these studies implicate the choice of chemotherapeutic agent as an explanation for the disparate outcomes. First, the use of fluorouracil as a potent and reliable inducer of intestinal injury is not unique to this study. Investigators of other growth factors with therapeutic efficacy in mucositis also have employed fluorouracil, which suggests that it does not possess a particular activity that renders its damage uniformly unamenable to the action of growth factors (7, 10, 36). Second, the use of melphalan, an alkylator, instead of the nucleoside analog fluorouracil by Robinson and co-workers (32) weakens any argument that the lack of benefit of EGF in chemotherapy-induced intestinal injury is primarily attributable to a specific chemotherapeutic agent. Finally, it is not likely that the negative findings of this study are the result of a distinct action of fluorouracil on EGF or its receptor because the Western blot analysis demonstrates that upregulation and activation of EGF receptor by exogenous EGF still occur after fluorouracil.

In addition to the studies that have been conducted in animals, Girdler and associates (11) tested the effects of a mouthwash containing EGF in humans that were receiving stomatotoxic chemotherapy. Girdler et al. (11) found that the EGF solution did not reduce the number or size of the oral ulcers compared with placebo. Although methodological considerations make direct comparisons to this study difficult, their conclusion (11) is consistent with the results of these experiments.

In summary, this study provides compelling evidence that EGF and its receptor do not have a critical role in the prevention or repair of chemotherapy-induced intestinal damage. Given the beneficial effects of EGF and its receptor in other models of intestinal injury, the mechanism for these findings deserves further investigation and may help to elucidate the pathophysiology of an important clinical problem.

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