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Am J Physiol Gastrointest Liver Physiol
278: G805–G810, 2000.

Aging alters gastric mucosal responses to epidermal growth factor and transforming growth factor-α

JERROLD R. TURNER,[1] LEI LIU,[2] SUZANNE E. G. FLIGIEL,[3]
RICHARD JASZEWSKI,[2,3] AND ADHIP P. N. MAJUMDAR[2,3,4,5]
[3]*Veterans Affairs Medical Center and Departments of* [2]*Medicine,* [1]*Pathology, and* [4]*Biochemistry and Molecular Biology and* [5]*Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201*

Turner, Jerrold R., Lei Liu, Suzanne E. G. Fligiel, Richard Jaszewski, and Adhip P. N. Majumdar. Aging alters gastric mucosal responses to epidermal growth factor and transforming growth factor-α. *Am J Physiol Gastrointest Liver Physiol* 278: G805–G810, 2000.—Administration of pharmacological doses of epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) in young rats stimulates gastric mucosal proliferation, but, in aged rats, the same treatment inhibits proliferation. This may be due to enhanced ligand-induced internalization of EGF receptor (EGFR). In support of this, we demonstrated that although a single injection of EGF (10 µg/kg) or TGF-α (5 µg/kg) in young (4–6 mo old) rats greatly increased membrane-associated EGFR tyrosine kinase activity, the same treatment slightly inhibited the enzyme activity in aged (24 mo old) rats. This treatment also produced a greater abundance of punctate cytoplasmic EGFR staining in gastric epithelium of aged rats, consistent with EGFR internalization. In vitro analyses demonstrated that exposure of isolated gastric mucosal cells from aged but not young rats to 100 pM TGF-α resulted in marked increases in intracellular EGFR tyrosine kinase activity and that induction of EGFR tyrosine kinase activity in mucosal membranes from aged rats occurred at doses 1,000-fold less than those required in young rats. Our data suggest that aging enhances sensitivity of the gastric mucosa to EGFR ligands. This may partly explain EGFR-mediated inhibition of gastric mucosal proliferation in aged rats.

epidermal growth factor receptor; epidermal growth factor receptor internalization; cell proliferation; tyrosine kinase

WE AND OTHERS HAVE SHOWN that aging results in increased gastrointestinal mucosal proliferative activity in Fischer 344 rats (2, 16, 17, 26, 28, 29). In gastric mucosa, this is also associated with increased activity of several tyrosine kinases, including the epidermal growth factor receptor (EGFR) (24, 32, 35, 41). EGFR is a 170-kDa transmembrane glycoprotein (8) with ligands including EGF, transforming growth factor-α (TGF-α), amphiregulin, and heparin-binding EGF (7, 8, 18, 42). Extracellular ligand binding leads to EGFR dimerization, activation of the receptor's intrinsic tyrosine kinase activity, and a signaling pathway resulting in cell proliferation (7, 8, 18, 36, 42). Our observation that the age-related rise in gastric mucosal proliferative activity is accompanied by a concomitant increase in mucosal EGFR tyrosine kinase suggests a role for EGFR in regulating gastric mucosal proliferation during aging (41).

EGFR ligands, most notably EGF and TGF-α, appear to be critically involved in regulating mucosal proliferation in the gastrointestinal tract, including the stomach (3, 34). However, the mitogenic responsiveness of the gastric mucosa to EGF changes with aging (23). We have demonstrated that although administration of pharmacological doses of EGF stimulates epithelial proliferation in young rats, the same treatment markedly inhibits epithelial proliferation in aged rats (23). These changes were accompanied by parallel alterations in overall tyrosine kinase activity and tyrosine phosphorylation of several membrane proteins (23).

The underlying biochemical mechanisms for age-related alterations in gastric mucosal response to exogenous EGF are not fully understood. We hypothesized that aging may enhance mucosal sensitivity to EGFR ligands so that low doses stimulate the EGFR-induced signal transduction pathway and enhance mucosal epithelial proliferation, whereas high doses inhibit these processes. To explore this, we evaluated age-related changes in mitogenic responsiveness of the gastric mucosa to TGF-α in parallel with EGFR tyrosine kinase activation and localization as well as EGFR internalization. The results are consistent with age-dependent increases in ligand-induced EGFR internalization, resulting in hypersensitivity to EGFR ligands in aged rats.

MATERIALS AND METHODS

Animals and collection of tissues. Male Fischer 344 rats, 4–6 and 22–24 mo old, were used. The animals were purchased from the National Institute on Aging (Bethesda, MD) at least 1 mo before the experiment and were fed with Purina rat chow and water ad libitum before the experiment.

Two in vivo experiments were performed. In the first set of experiments, groups of three young (4–6 mo old) and three aged (22–24 mo old) rats were implanted with subcutaneous osmotic mini-pumps (Alza, Palo Alto, CA) that delivered either TGF-α (100 $ng \cdot kg^{-1} \cdot h^{-1}$) or 0.9% NaCl (controls) for 4 days. During this period, animals receiving TGF-α and 0.9% NaCl were provided with identical amounts of daily chow to

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minimize differences due to intake. All animals were fasted (water was supplied) during the last 16 h of each experiment. Each animal was killed, and the stomach was removed, cut along the greater curvature, and washed thoroughly in cold 0.9% NaCl. The oxyntic gland area was removed, and small portions were fixed in buffered 10% neutral formalin and used for studies of mucosal proliferation.

In the second in vivo experiment, groups of young and aged rats that had been fasted overnight were injected intraperitoneally with EGF (10 μg/kg), TGF-α (5 μg/kg in 10 mM acetic acid), or vehicle controls. These animals were killed 30 min later, and their stomachs were removed and treated as stated above. Additionally, mucosal scrapings from the oxyntic gland area were stored at −80°C.

In vitro experiments were performed with freshly isolated gastric mucosal cells obtained from overnight-fasted young and aged rats. Cells were isolated by a slight modification of the procedure described by Kinoshita et al. (21). Briefly, the stomach was removed, everted, and filled with 5 ml of Pronase solution (5 mg/ml) in *buffer A* (0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 50 mM HEPES, pH 7.2, 20 mM $NaHCO_3$, 2% BSA, and 2 mM EDTA). The gastric bag was then incubated in Pronase-free *buffer A* at 37°C for 30 min under constant O_2 bubbling. The gastric bags were then transferred to *buffer B* (0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 50 mM HEPES, pH 7.2, 20 mM $NaHCO_3$, 2% BSA, 1 mM $CaCl_2$, and 1.5 mM $MgCl_2$) and gently agitated at 24°C for 90 min. The released cells were recovered by centrifugation at 200 *g* for 5 min and resuspended in *buffer A*. Typical recovery was 10–12 x 10^6 cells per adult rat stomach. Trypan blue exclusion was >95%. Cell composition was similar in preparations from young and aged animals. Aliquots of cells in Pronase-free *buffer A* were equilibrated at 37°C for 15 min and subsequently incubated in the absence or presence of 100 pM TGF-α.

Mucosal histology. Formalin-fixed gastric tissues were dehydrated in graded alcohol and embedded in paraffin to yield full-thickness mucosal sections. Serial sections (4 μm thick) were stained with hematoxylin and eosin.

Determination of proliferating cell nuclear antigen immunoreactivity. Serial sections (4 μm thick) were deparaffinized and incubated at ambient temperature with anti- proliferating cell nuclear antigen (PCNA; monoclonal antibody, Glostrup) for 30 min at 1:50 dilution. The avidin-biotin technique was then performed with matched components (secondary biotinylated antibody and avidin-peroxidase complex) from the DAKO labeled streptavidin-biotin system (Carpinteria, CA) according to the manufacturer's suggested protocol. Amino ethyl carbazole was used as chromagen to localize PCNA-positive cells. All slides were lightly counterstained with Harris' hematoxylin and examined by a pathologist blinded to sample coding. At least 10 well-oriented crypts on each slide and 5 slides from each sample were examined under high power. At least 750 cells/slide were counted using a ×40 objective.

Immunofluorescent staining of EGFR. Serial sections (4 μm thick) of gastric tissues were collected on poly-L-lysine-coated slides. After deparaffinization and rehydration, sections were blocked with 10% chick serum, 10% porcine serum, and 0.5% BSA in PBS for 1 h at room temperature. After being washed with PBS twice, the slides were incubated overnight at 4°C with 2 μg/ml polyclonal rabbit anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit γ-globulin (Sigma Chemical, St. Louis, MO) in PBS was used as a negative control. After extensive washing in PBS, the slides were incubated with 20 μg/ml FITC-conjugated-F(ab′)$_2$ fragment of anti-rabbit IgG (Jackson Immunolabs, West Grove, PA) for 1 h at room temperature. After being washed in PBS, slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The slides were examined using a Zeiss LSM 310 imaging system.

Tyrosine kinase activity of EGFR. Tyrosine kinase activity was determined in the surface membrane and intracellular fractions as previously described (20, 27). Briefly, aliquots of frozen mucosal scrapings or freshly isolated gastric mucosal cells were homogenized in homogenizing buffer [HEPES, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM 1,10-phenanthroline] and centrifuged at 30,000 *g* for 30 min to obtain crude membrane (30,000 *g* pellet) and intracellular (30,000 *g* supernatant) fractions. The surface membrane fraction was resuspended in homogenizing buffer. After determination of protein content using the Bio-Rad standard protein assay kit (Hercules, CA), aliquots of surface membrane and intracellular fractions containing 350 μg of protein were diluted with an equal volume of radioimmunoprecipitation assay (RIPA) buffer (20 mM sodium phosphate, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM 1,10-phenanthroline). The samples were incubated overnight at 4°C with 1 μg of polyclonal antibody to EGFR (UBI, Lake Placid, NY). The immune complexes were precipitated with heat-denatured Pansorbin, washed several times with RIPA/homogenizing buffer. The immunoprecipitates were resuspended in 30 μl of homogenizing buffer containing 0.1% Triton X-100 and assayed for tyrosine kinase activity by measuring ^{32}P incorporation from [γ-^{32}P]ATP into acid-denatured enolase (20, 27). In all immunoprecipitation studies, protein concentration was standardized among the samples.

Statistical analysis. Where applicable, results were evaluated with Student's *t*-test for unpaired samples.

RESULTS

EGF and TGF-α induce proliferation in gastric mucosa of young rats but not aged rats. We have previously shown (23) that supraphysiological doses of EGF inhibit epithelial proliferation in the gastric mucosa of aged rats. To determine whether this effect was specific to EGF or could be reproduced with other EGFR ligands, rats were treated with TGF-α by continuous infusion over 4 days. Unlike EGF, which is primarily synthesized in the salivary gland, TGF-α is synthesized in the gastric mucosa (3). Thus TGF-α may be a more physiologically relevant ligand for gastric mucosal EGFR. The effect of TGF-α is similar to that previously reported for EGF (23). In young rats, infusion of TGF-α markedly increased proliferative activity in the gastric mucosa, as evidenced by a 65% increase in PCNA-immunoreactive cells relative to controls ($P < 0.001$; Fig. 1). In contrast, TGF-α induced a 64% decline in PCNA-immunoreactive gastric mucosal cells in aged rats ($P < 0.001$; Fig. 1). In the absence of TGF-α treatment, aged rats demonstrated a 50% increase in PCNA-immunoreactive gastric epithelial cells relative to young rats ($P < 0.001$; Fig. 1).

EGF and TGF-α induce distinct patterns of EGFR redistribution in gastric mucosa of young and aged rats. We considered the possibility that the age-related change in mitogenic response to EGF and TGF-α could be due to increased internalization or degradation of

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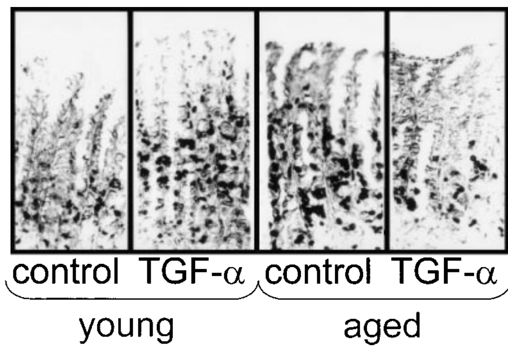

Fig. 1. Proliferating cell nuclear antigen (PCNA) immunoreactivity increases in gastric mucosa of young rats but decreases in aged rats after 4 days of infusion with transforming growth factor-α (TGF-α) (100 ng/kg) or 0.9% NaCl (controls). PCNA-positive cells/gastric gland: young control rats, 6.8 ± 1.3; young TGF-α-treated rats, 11.2 ± 1.5 ($P < 0.001$); aged control rats, 14 ± 0.9; aged TGF-α-treated rats, 5 ± 0.9 ($P < 0.001$, $n = 3$).

EGFR in gastric epithelium of aged rats. We evaluated this hypothesis both morphologically and biochemically. Because ligand-induced activation and subsequent internalization of EGFR are early events in the EGFR signaling cascade (10, 42), gastric mucosa was evaluated 30 min after a single injection of EGF or TGF-α (Fig. 2). Immunolocalization of EGFR of control young rats showed that the majority of EGFR was localized to the apical membrane (Fig. 2*A*). The overall EGFR immunostaining appeared to be less intense than in the control aged rats. Administration of EGF or TGF-α to young rats induced the appearance of EGFR-containing punctate vesicular structures in the basal cytoplasm. Additionally, TGF-α induced the appearance of diffuse apical cytoplasmic EGFR staining. In control aged rats (Fig. 2*B*) the overall EGFR staining intensity was increased relative to young rats. EGFR was localized to the apical membrane and to basal cytoplasmic vesicular structures. These cytoplasmic structures were similar to those seen in EGF- or TGF-α-treated young rats. However, the staining intensity, number, and size of these basal structures was greater than that seen in control or EGF- or TGF-α-treated young rats. Administration of EGF or TGF-α to aged rats induced marked increases in EGFR detected within these basal vesicular structures. These data support the hypothesis that internalization of EGFR occurs at an increased basal level in the gastric mucosa of aged rats as opposed to young rats. The data also suggest that ligand-induced internalization of EGFR is more rapidly triggered in aged rats relative to young rats. We considered the possibility that these changes might be due to altered cell composition in the oxyntic mucosa of young and aged animals, but no significant differences in relative density of foveolar, chief, or parietal cells were apparent on examination of hematoxylin and eosin-stained sections.

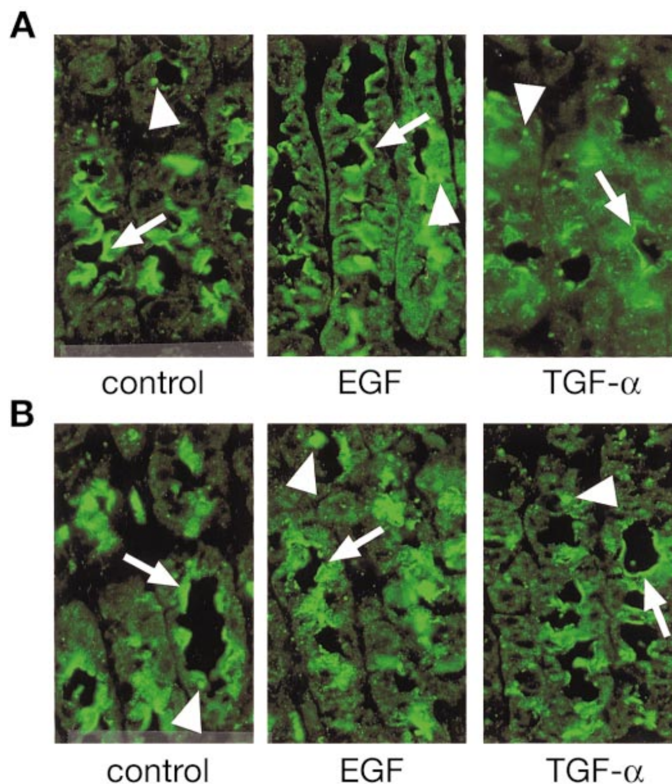

Fig. 2. Epidermal growth factor (EGF) receptor (EGFR) is more internalized in gastric mucosa of young and aged rats 30 min after a single intraperitoneal injection of EGF (10 µg/kg), TGF-α (5 µg/kg), or vehicle. *A*: Immunolocalization of EGFR of young control rats showed that majority of EGFR was localized to apical membrane (arrows). EGF or TGF-α resulted in appearance of EGFR-containing punctate vesicular structures in basal cytoplasm (arrowheads). *B*: In control aged rats EGFR was localized to apical membrane (arrows) and to cytoplasmic vesicular structures (arrowheads). EGF or TGF-α caused increases in EGFR within vesicular structures.

Response of gastric epithelial EGFR tyrosine kinase activity to in vivo EGF and TGF-α is greater in young than in aged rats. To determine whether the apparent ligand-induced EGFR internalization correlated with altered activation, EGFR tyrosine kinase activity was measured in membrane preparations from gastric epithelium of young and aged rats 30 min after administration of EGF or TGF-α. In control rats, total EGFR tyrosine kinase activity of aged rats was 417% of that in young controls (Fig. 3). Administration of EGF and TGF-α to young rats induced 483% and 1,100% increases, respectively, in total EGFR tyrosine kinase activity (Fig. 3). In contrast, EGF had no effect and TGF-α produced a 21% decrease in total EGFR tyrosine kinase activity in aged rats (Fig. 3). These data are consistent with increased basal activation of EGFR in aged rats. The data also support the hypothesis that ligation of EGFR in aged rats results in downregulation of membrane receptor kinase activity, perhaps via internalization, whereas receptor ligation induces activation of EGFR tyrosine kinase activity in young rats.

Isolated gastric epithelial cells from aged rats internalize EGFR tyrosine kinase activity more rapidly than gastric epithelial cells from young rats. To further characterize the proposed enhanced internalization of EGFR in aged rats, surface membrane and intracellular fractions were isolated from gastric epithelial cells stimulated in vitro. When epithelial cells isolated from young rats were exposed to 100 pM TGF-α, EGFR tyrosine kinase activity increased steadily between 5 and 20 min, both in surface membrane and intracellular fractions. Fifty percent of total EGFR kinase activ-

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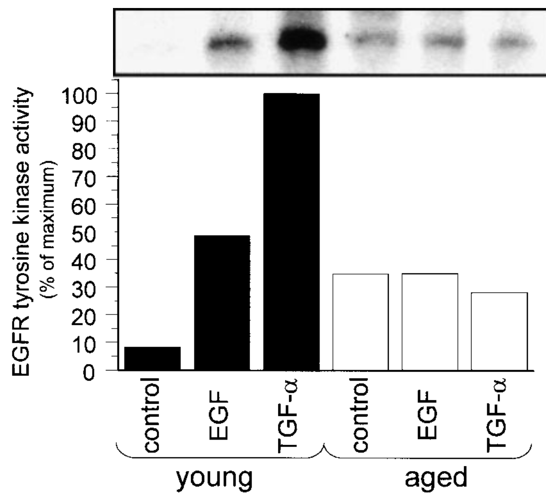

Fig. 3. EGFR tyrosine kinase activity increases in gastric mucosal membranes from young rats, but not aged rats, 30 min after a single intraperitoneal injection of EGF (10 μg/kg), TGF-α (5 μg/kg), or an equivalent volume of vehicle (control). Bars (*bottom*) show EGFR tyrosine kinase activity as determined by densitometry of SDS-PAGE (*top*). Values were normalized to density of darkest band (young rats stimulated with TGF-α); $n = 3$ separate experiments.

ity was present in the intracellular fraction at 5 min after TGF-α stimulation. This increased to 69% and 67% of total EGFR kinase activity after 10 and 20 min of stimulation, respectively (Fig. 4). In contrast, although total EGFR tyrosine kinase activity increased at each interval in epithelial cells from aged rats, the intracellular fraction of EGFR kinase activity was 73%, 71%, and 84% at 5, 10, and 20 min, respectively (Fig. 4). Thus at each interval after TGF-α stimulation the intracellular fraction of EGFR tyrosine kinase activity was greater in aged than in young rats. These data support the hypotheses that gastric epithelial cells from aged rats have increased basal EGFR activation compared with cells from young rats and that a greater

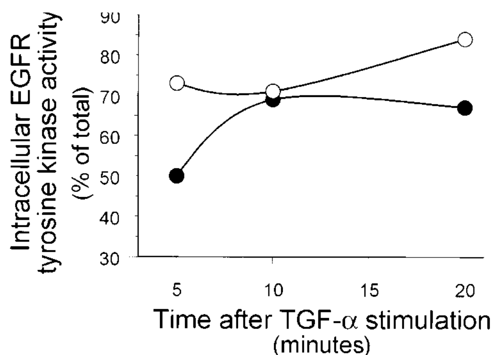

Fig. 4. TGF-α triggers EGFR internalization in gastric epithelial cells from young and aged rats. Gastric epithelial cells from young (●) or aged (○) rats were incubated in presence of 100 pM TGF-α for 5, 10, or 20 min. Cells were then fractionated into surface membrane and intracellular fractions. EGFR was immunoprecipitated, and EGFR tyrosine kinase activity was determined. Each point represents fraction of EGFR tyrosine kinase activity that was intracellular. At each time point, total EGFR tyrosine kinase activity in intracellular fractions from aged animals was significantly greater than that of young animals (old-to-young ratio = 1.9 after 20 min of TGF-α stimulation).

fraction of EGFR is intracellular in aged rats after TGF-α stimulation.

Gastric mucosal membranes from aged rats are more sensitive to TGF-α stimulation than those from young rats. To determine whether aging enhances responsiveness of the gastric mucosa to the EGF family of peptides, we examined age-related changes in EGFR tyrosine kinase activity in mucosal membrane preparations from young and aged rats in response to increasing concentrations of TGF-α. As shown in Fig. 5, EGFR tyrosine kinase activity in mucosal membranes from aged rats was maximally stimulated with a dose of 10 pM TGF-α. Doses above 10 pM inhibited the maximal stimulatory action of the peptide. The results show a biphasic response of the gastric mucosa of aged rats to TGF-α. However, no such biphasic response was observed in young rats. In fact, EGFR tyrosine kinase activity in mucosal membranes from young rats was not significantly affected until the TGF-α concentration was elevated to 1 nM, a dose that was 1,000-fold greater than the lowest dose (1 pM) that significantly stimulated EGFR tyrosine kinase in mucosal membranes from aged rats. Our current results show that gastric mucosal sensitivity to TGF-α is considerably higher in aged than young rats. This is consistent with in vivo "priming" of EGFR in aged animals.

DISCUSSION

Cells of the gastrointestinal mucosa undergo a constant process of renewal. In normal adults, this reflects a balance between exfoliation of surface cells and proliferation of precursor cells. However, the proliferative rate is not constant throughout life. In fact, accumulating data suggest that gastrointestinal epithelial cells undergo age-dependent changes in their proliferative rate (26, 28, 29, 34). For example, we have shown that DNA synthesis and thymidine kinase activity, as measures of gastric epithelial proliferative activity, are elevated during the first 2 wk of life and then decrease dramatically over the next 2–3 wk (30). More recently, we and others have shown that gastric (24, 28, 35), small intestinal (16, 17), and large intestinal (15)

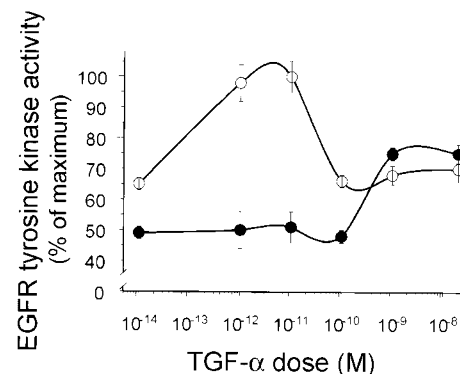

Fig. 5. Gastric mucosal membranes from aged rats are more sensitive to TGF-α than mucosal membranes from young rats. Mucosal membranes from young (●) or aged (○) rats were stimulated with indicated doses of TGF-α for 15 min. EGFR tyrosine kinase activity was determined over a 15-min interval and normalized to peak values (10 pM TGF-α in aged rats).

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mucosal proliferative activity increase with advancing age. The observation reported herein that more PCNA-immunoreactive cells are present in the gastric mucosa of aged rats than in young rats also supports the contention that aging is associated with increased gastric mucosal proliferative activity.

Over the past three decades, numerous reports have appeared suggesting that gastrointestinal hormones and peptides, most notably gastrin, bombesin, EGF, and TGF-α, regulate mucosal proliferation throughout the gastrointestinal tract, including the stomach, both in vivo and in vitro (3–6, 11–14, 18, 22, 30, 37–40, 43). However, increases in gastric mucosal proliferative activity in aged rats cannot be attributed to increased responsiveness to any of these hormones and peptides. In fact, aging is associated with a loss of gastric mucosal responsiveness to the trophic activities of both gastrin and bombesin (25, 31), and EGF inhibits mucosal proliferative activity in aged rats (23). Our current observation that pharmacological doses of TGF-α, which stimulate gastric epithelial proliferation in young rats, inhibit gastric epithelial proliferation in aged rats is analogous to our previous observations using EGF (23). Clearly, these results suggest that large doses of either EGF or its structural and functional analog TGF-α inhibit gastric epithelial proliferation in aged rats. Similar observations have been reported for the human breast cancer cell line MDA-MB-468 and the human epidermoid carcinoma cell line A431, each of which overexpresses EGFR (1, 9, 19). Interestingly, aging is also associated with increased expression of EGFR in the gastric mucosa (41). Together, the results suggest an inverse relationship between overexpression of EGFR and mitogenic response to EGF and TGF-α. On the basis of these observations, we postulated that the inhibition of gastric mucosal proliferative activity in aged gastric mucosa in response to pharmacological doses of EGF or TGF-α could be the result of increased ligand-induced internalization or degradation of EGFR. Our current data appear to support such a contention. We have observed that in aged rats, but not in young rats, a single injection of a pharmacological dose of either EGF or TGF-α produced a marked rise in the relative abundance and intensity of basal vesicular cytoplasmic EGFR staining within the gastric epithelium. This result suggests that the gastric epithelium of aged rats responds to pharmacological doses of EGFR ligands with an increased internalization response relative to young rats. We considered the possibility that differential distribution of exogenous EGF or TGF-α could explain the differences between young and old rats but concluded that this was unlikely since similar differential responses were noted in vitro. Although we did not evaluate the extent of EGFR-ligand complex degradation biochemically, the progressive increases in intracellular EGFR tyrosine kinase activity observed in vitro also support the conclusion that the internalization response in aged rats is exaggerated. The in vitro studies also demonstrated that 100 pM TGF-α induced much greater and more rapid increases in intracellular EGFR tyrosine kinase activity in gastric epithelial cells from aged rats than did similar stimulation of gastric epithelial cells from young rats. Although the regulatory mechanisms for the increased internalization of EGFR in the gastric mucosa of aged rats in response to exogenous EGF or TGF-α remain to be elucidated, our data suggest that aging may be associated with increased sensitivity of the EGFR to its ligands. It is also possible that some of the differences may be due to altered cytoskeletal responsiveness of epithelial cells from young and aged rats, as polyamine depletion has been recently reported to alter both actin structure and EGFR function in the IEC-6 cell line (33). Consistent with this, we showed that the concentration of TGF-α needed to induce maximal in vitro stimulation of EGFR tyrosine kinase activity in gastric mucosal membranes from aged rats was 1,000-fold less than that required in young rats.

In conclusion, the data presented in this study demonstrate that aging is associated with altered responsiveness of gastric mucosal epithelium to pharmacological doses of the EGFR ligands EGF and TGF-α. These peptides stimulate proliferative activity in gastric epithelial cells from young rats but cause inhibition of proliferation in aged rats. The greater extent of EGFR internalization observed in the gastric mucosa of aged rats as opposed to that seen in young rats suggests that rapid internalization of the ligand-receptor complex may partly contribute to the inhibition of mucosal proliferative activity in aged rats. Thus altered responses of the gastric epithelial EGFR occur with aging and may, in part, explain age-related changes in epithelial proliferation and responses to peptide growth factors.

We thank Linda Mayernik for expert assistance with confocal microscopy.

This study was supported by the National Institute on Aging and the Department of Veterans Affairs (A. P. N. Majumdar) and National Institute of Diabetes, Digestive, and Kidney Diseases Grants DK-02503 and DK-56121 (J. R. Turner) and Institute of Chemical Toxicology of Wayne State University Grant NIH P30-ES06639.

Address for reprint requests and other correspondence: A. P. N. Majumdar, Research Service-151, Veterans Affairs Medical Center, 4646 John R, Detroit, MI 48201 (E-mail: a.majumdar@wayne.edu).

Received 15 September 1999; accepted in final form 2 December 1999.

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