Induction of transcriptional activity of AP-1 and NF-κB in the gastric mucosa during aging

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Xiao, Zhi-Qiang, and Adhip P. N. Majumdar. Induction of transcriptional activity of AP-1 and NF-κB in the gastric mucosa during aging. Am J Physiol Gastrointest Liver Physiol 278: G855–G865, 2000.—Although aging enhances expression and tyrosine kinase activity of epidermal growth factor receptor (EGFR) in the gastric mucosa, there is no information about EGFR signaling cascades. We examined the age-related changes in mitogen-activated protein kinases (MAPKs) [extracellular signal-related kinases (ERKs), c-Jun NH2-terminal kinases (J Nks), and p38], an EGFR-induced signaling cascade, and activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcriptional activity in the gastric mucosa of 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats. AP-1 and NF-κB transcriptional activity in the gastric mucosa rose steadily with advancing age. This can be further induced by transforming growth factor-α. The age-related activation of AP-1 and NF-κB in the gastric mucosa was associated with increased levels of c-j un, c-Fos, and p52, but not p50 or p65. Total and phosphorylated IκBα levels in the gastric mucosa were unaffected by aging. Aging was also associated with increased expression of p21Waf1. This suggests that the age-related rise in gastric mucosal proliferative activity could partly be the result of the enhanced progression of cells through the G1 phase.

Although the responsible molecular mechanisms for the age-related rise in mucosal proliferative activity are poorly understood, we (37, 46) have observed that, at least in the gastric mucosa, aging is associated with increased expression and activation of certain tyrosine kinases, most notably the epidermal growth factor receptor (EGFR), the common receptor for EGF and transforming growth factor-α (TGF-α). Numerous studies (4, 6, 19, 42) have demonstrated that the EGF family of peptides, particularly EGF and TGF-α, stimulate mucosal proliferative activity in much of the gastrointestinal tract, including the stomach. Both EGF and TGF-α initiate their mitogenic action by activating the intrinsic tyrosine kinase activity of their receptor, thereby initiating the EGFR signaling process (41, 42, 47). In view of this, we (37, 46) postulated that induction of the EGFR signaling pathway may partly be responsible for the age-related rise in gastric mucosal proliferative activity. Additionally, we (46) have observed that the relative concentration of membrane-bound forms of TGF-α as well as mRNA expression of the peptide are also markedly higher in the gastric mucosa of aged rats than in young rats. This suggests that TGF-α, which, unlike EGF, is synthesized in the gastric mucosa, may stimulate mucosal EGFR tyrosine kinase through an autocrine/juxtocrine mechanism (46).

Activation of EGFR initiates a series of signaling events through phosphorylation of interacting proteins, which in turn transmit the signal to the nucleus (41, 42, 47). The details of the signaling events leading to stimulation in mucosal proliferative activity during advancing age are yet to be elucidated. However, a large body of evidence suggests that the mitogen-activated protein kinase (MAPK) signaling pathways, which regulate cellular growth and differentiation, respond to EGFR activation (18, 40, 43). At least three distinct families of MAPKs are present in mammalian cells: the p42/44 extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal/stress-activated kinases (J Nks/SAPks), and p38 (39). It has been suggested that ERKs are primarily responsive to cell proliferation signals, whereas J Nks and p38 respond to cellular stress (10, 21, 23). Once activated, MAPKs translocate to the nuclei of target cells, where they bind specific DNA elements of the regulatory gene and induce transcription of new proteins. Transcription factors, such as AP-1 and NF-κB, play a crucial role in the regulation of the expression of many genes involved in cell growth and differentiation. AP-1 and NF-κB are composed of several dimers of different subunits, which can interact with specific DNA sequences in the promoter regions of genes. The components of AP-1 and NF-κB are found in many cell types and are involved in a wide variety of biological processes, including cell growth and differentiation, immune responses, and cancer.

RESULTS FROM THIS AND OTHER LABORATORIES (2, 16, 17, 19, 29, 31, 32, 36, 37) have demonstrated that in the Fischer 344 rat model, aging is associated with increased mucosal proliferative activity in various tissues of the gastrointestinal tract, including the stomach. In the gastric mucosa, this is evidenced by increased labeling index, DNA synthesis, and thymidine kinase and ornithine decarboxylase (ODC) activities (29, 31, 32, 36). More recently, we (49) have demonstrated that aging is also associated with increased expression of cyclin-dependent kinase-2, accompanied by decreased expression of p21Waf1. This suggests that the age-related rise in gastric mucosal proliferative activity could partly be the result of the enhanced progression of cells through the G1 phase.

Although the responsible molecular mechanisms for the age-related rise in mucosal proliferative activity are poorly understood, we (37, 46) have observed that, at least in the gastric mucosa, aging is associated with increased expression and activation of certain tyrosine kinases, most notably the epidermal growth factor receptor (EGFR), the common receptor for EGF and transforming growth factor-α (TGF-α). Numerous studies (4, 6, 19, 42) have demonstrated that the EGF family of peptides, particularly EGF and TGF-α, stimulate mucosal proliferative activity in much of the gastrointestinal tract, including the stomach. Both EGF and TGF-α initiate their mitogenic action by activating the intrinsic tyrosine kinase activity of their receptor, thereby initiating the EGFR signaling process (41, 42, 47). In view of this, we (37, 46) postulated that induction of the EGFR signaling pathway may partly be responsible for the age-related rise in gastric mucosal proliferative activity. Additionally, we (46) have observed that the relative concentration of membrane-bound forms of TGF-α as well as mRNA expression of the peptide are also markedly higher in the gastric mucosa of aged rats than in young rats. This suggests that TGF-α, which, unlike EGF, is synthesized in the gastric mucosa, may regulate mucosal EGFR tyrosine kinase through an autocrine/juxtocrine mechanism (46).

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to the nucleus, where they activate transcription factors (20).

Among the transcription factors, activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) have been extensively investigated since they appear to be critically involved in regulating the expression of a variety of genes that participate in growth-related processes (3, 20, 24). NF-κB is thought to be involved in regulating the expression of genes required for inflammatory responses, for suppression of apoptosis as well as for controlling proliferation (4, 12). AP-1, which is also involved in regulating cell proliferation, responds to many stimuli, including growth factors and cytokines (1, 20). Furthermore, subunits of AP-1 and NF-κB are able to cross talk, and both factors may play a role in cell transformation (7, 44). In view of the evidence indicating a role for AP-1 and NF-κB in regulating cellular growth, we have examined the age-related changes in AP-1 and NF-κB transcriptional activity in the gastric mucosa. The primary objective was to determine whether the age-related rise in gastric mucosal proliferative activity would also be accompanied by increased activation of AP-1 and NF-κB transcription factors. Because MAPKs play a key role in activating transcription factors through phosphorylation, we also examined the activity of different MAPKs in the gastric mucosa during aging. Our current data show that aging is associated with a marked increase in expression and activation of AP-1 and NF-κB in the gastric mucosa and that these changes are accompanied by increased ERK and JNK1 activity, but not p38 MAPK activity. In addition, we have also demonstrated that in isolated gastric mucosal cells, TGF-α markedly stimulates the transcriptional activity of AP-1 and NF-κB.

METHODS

Reagents. Double-stranded oligonucleotide probes containing the consensus sequences of NF-κB and AP-1 as well as the...
T4 polynucleotide kinase were from Promega (Madison, WI). The sequences of the oligonucleotides were as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and AP-1, 5′-CGC TTG ATG AGT CAG CCG GAA-3′. Poly(dl-dC)-poly(dl-dC) and protein G-Sepharose were obtained from Pharmacia Biotech (Piscataway, NJ). [γ-32P]ATP (3,000 Ci/mmol) was from NEN Life Science (Boston, MA). Polyclonal rabbit antibodies to NF-κB p50, p52, and p65, J κ B, c-j un, c-Fos, ERK1, and ERK2 as well as to c-j un (amino acids 1–79) substrate were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibodies to p38, phospho-p38 (Thr180/Tyr182), phospho-ERK1, phospho-ERK2 (Thr202/Tyr204), IκBα, and phospho-IκBα (Ser32) were from New England Biolabs (Beverly, MA). Goat anti-rabbit IgG conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL) were obtained from Amersham (Arlington Heights, IL). Immobilon-P nylon membrane was from Millipore (Bedford, MA), and X-Omat AR film was from Eastman Kodak (Rochester, NY). Concentrated protein assay dye reagent was from Bio-Rad (Hercules, CA). Molecular weight marker, myelin basic protein (MBP), and DMEM/F-12 medium were from GIBCO BRL (Grand Island, NY). Recombinant human TGF-α was a product of Calbiochem (La J ola, CA). All other reagents were of molecular biology grade and were from either Sigma Chemical or Fisher Scientific.

Animals and collection of gastric mucosal epithelial cells. Male Fischer 344 rats aged 4–6 (young), 12–24 (middle aged), and 22–24 (old) mo were used. The animals were obtained from the National Institute on Aging (Bethesda, MD), 2 mo before the experiment. During this period, they had access to Purina rat chow and water ad libitum. Animals were fasted overnight before the experiments.

All experiments were performed utilizing freshly isolated gastric mucosal cells. Cells were isolated from overnight fasted rats by a slight modification of the procedure described by Kinoshita et al. (22). Briefly, the contents of the stomach were washed out with PBS. After being transformed into inside-out gastric bags, they were filled with 5 ml of 3 mg/ml Pronase solution in buffer A (0.5 mM NaH2PO4, 1.0 mM Na2HPO4, 70 mM NaCl, 5.0 mM KCl, 11 mM glucose, 50 mM HEPES, pH 7.2, 20 mM NaHCO3, 2 mM EDTA, and 5% BSA). The filled gastric bags were incubated in Pronase-free buffer A at 37°C for 30 min. The gastric bags were then transferred into buffer B (containing 1.0 mM CaCl2 and 1.5 mM MgCl2 instead of EDTA in buffer A) and gently agitated by a magnetic stirrer at room temperature for 1 h. The epithelial cells, dispersed in buffer B, were collected by centrifugation at 500 g for 5 min. The cell pellets were immediately processed for nuclear or total cell extracts.

Preparation of nuclear extracts. Nuclear extracts were prepared by a slight modification of the method described by Dignam et al. (8) and Gupta et al. (14). Briefly, cells were resuspended in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 5 µg/ml of aprotinin, pepstatin A, and leupeptin) and incubated for 10 min at 4°C. Swollen cells were homogenized with 10 or more slow up-and-down strokes in a glass Dounce homogenizer and centrifuged at 3,300 g for 15 min at 4°C. The pelleted nuclei were washed once with ice-cold low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM PMSF, 1.0 mM DTT, 0.2 mM EDTA, and 5 µg/ml of aprotinin, pepstatin A, and leupeptin) by centrifuging at 10,000 g for 15 min at 4°C. The nuclei were resuspended in ice-cold low-salt buffer, and nuclear protein was released by adding an ice-cold high-salt buffer (same as the low-salt buffer, except that it contained 1.2 M KCl) drop by drop to a final concentration of 0.4 M KCl. The samples were rotated at 4°C for 30 min. The nuclear extracts were recovered by centrifugation at 25,000 g for 30 min at 4°C and stored at –80°C in small aliquots.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMS A) was utilized to determine the transcriptional activity of NF-κB and AP-1 by assaying the extent of binding of nuclear extracts to NF-κB and AP-1 consensus sequences as described by Gupta et al. (14). Briefly, probes containing the consensus sequences of NF-κB or AP-1 were labeled with [γ-32P]ATP, using T4 polynucleotide kinase according to the protocol provided by Promega. Labeled oligonucleotides were purified by chromatography through a Sephadex G-25 spin column. For DNA-protein binding reactions, 5 or 10 µg of nuclear protein and 2 µg of poly(dl-dC)-poly(dl-dC) were preincubated in 20 µl binding buffer (10 mM HEPES, pH 7.5, 4% glycerol, 1.0 mM MgCl2, 50 mM KCl, 0.5 mM EDTA, and 1.0 mM DTT) for 15 min at 4°C and then 300,000 counts/min of radiolabeled probe were added. Reactions were further incubated for 30 min at room temperature. In NF-κB EMSA, the binding buffer contained 0.05% Nonidet

Fig. 3. Representative Western blots showing changes in c-j un (A) and c-Fos levels (B) in gastric mucosal nuclei during aging. Aliquots of nuclear extracts (20 µg protein) from freshly isolated gastric mucosal cells from 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats were subjected to Western blot analysis. Relative changes in c-j un and c-Fos levels among the 3 age groups, as determined by densitometric analysis, are shown in the histogram.
The resulting products were separated by 6–7% native polyacrylamide gel containing 0.25× TBE (89 mM Tris, pH 8.3, 89 mM boric acid, and 2 mM EDTA) with 0.25× TBE as the running buffer. Gels were dried and exposed to film at −80°C with intensifying screens. Signals on the film were quantitated by densitometry using ImageQuant image analysis system (Storm optical scanner, Molecular Dynamics, Sunnyvale, CA). Competition was performed by adding the respective nonradioactive oligonucleotide probes to the reaction mixture in a 50-fold molar excess. All assays were repeated at least three times using nuclear extracts from different rats for each age group.

In some experiments, TGF-α-induced changes in DNA binding activity of AP-1 and NF-κB were examined in isolated gastric mucosal cells. Briefly, freshly isolated mucosal cells from young and aged rats were washed once with serum-free DMEM/F-12 medium and then suspended in serum-free DMEM/F-12. Aliquots of mucosal cells were then incubated in the absence or presence of 1 nM TGF-α at 37°C for 20 min. Nuclear extracts were immediately prepared and used for assessing AP-1 and NF-κB binding activities by EMSA as described above.

Supershift EMSA. In supershift assays, EMSAs were performed as described above except that the binding reaction contained 3 µg of polyclonal antibodies to components of NF-κB (p50, p52, or p65) or AP-1 (c-Jun or c-Fos).

Western immunoblot analysis. This was performed according to our standard protocol (35). In all analyses, protein concentration was standardized among the samples. Briefly, nuclear proteins (20 µg) were separated by SDS-PAGE and then electroblotted to Immobilon-P nylon membranes. The membranes were blocked overnight with 5% nonfat dried milk in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM NaCl, and 0.1% Tween 20), followed by 3 h of incubation with the primary antibodies (1:1,000–1:1,500 dilution) in TBS-T buffer containing 5% nonfat dried milk or 1% BSA at room temperature. After being washed three times with TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution) for 1 h at room temperature. Protein bands were visualized using the ECL detection system and quantitated by densitometry. All of the Western immunoblots were performed at least three times using nuclear extracts or total cell lysates from different rats for each age group.

MAPK assay. Immunocomplex kinase assays were performed as described previously (14, 48). Briefly, the cell pellets were washed with ice-cold PBS and lysed at 4°C in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM...
RESULTS

To determine whether aging affects AP-1 and NF-κB transcriptional activity in the gastric mucosa, we performed EMSA to examine the extent of binding of nuclear extracts from isolated mucosal cells from rats aged 4–6 (young), 12–14 (middle aged), and 22–24 (old) mo to the consensus sequence of either AP-1 or NF-κB.

AP-1 DNA binding activity in the gastric mucosa of 12- to 14- and 22- to 24-mo-old rats was increased by ~60% and 190%, respectively, compared with their 4- to 6-mo-old counterparts (Fig. 1). No appreciable binding of nuclear extracts to the AP-1 consensus was detected in the presence of 50-fold molar excess of unlabeled oligonucleotide probe (Fig. 1). Because AP-1 is composed of members of the Jun and Fos families (20), supershift EMSA was performed with antibodies against Jun and Fos to detect their presence in the DNA-protein complexes. Antibodies against either Jun or Fos completely supershifted the DNA complexes formed with different components of NF-κB. No such supershift occurred in the presence of control rabbit serum.

NF-κB, a member of the Rel family of transcription factors, consists of five members (c-Rel, p50, p65, p52, and RELB), which form homo- or heterodimers (3). Different subunit combinations have different func-

Fig. 5. Representative autoradiograph from supershift EMSA analysis showing presence of different NF-κB components (p50, p52, and p65) in nuclear extracts from freshly isolated gastric mucosal cells from 22- to 24-mo-old rats. Aliquots of nuclear extracts (10 µg protein) from freshly isolated gastric mucosal cells were subjected to supershift EMSA analysis containing 10 µg of protein and 3 µg antibodies against p50, p52, or p65 components of NF-κB and 32P-labeled oligonucleotide probe with consensus sequence of NF-κB.
tions in regulating transcription (3). However, the predominant NF-κB activator of transcription is a p50/p65 heterodimer. To evaluate the involvement of different members of the Rel family in modulating NF-κB DNA binding activity in the gastric mucosa during aging, levels of p50, p52, and p65 subunits in the gastric mucosa of 4- to 6-, 12- to 14-, and 22- to 24-mo-old rats were assayed by Western blot analysis (Fig. 6). In the gastric mucosa, p52 levels rose steadily with advancing age, revealing 90% and 150% higher values in 12- to 14- and 22- to 24-mo-old rats, respectively, compared with their younger counterparts. In contrast, levels of p50 and p65 in the gastric mucosa were not significantly affected by aging.

IκBα, the most widely studied member of the IκB protein family, has been shown to play a key role in regulating nuclear import of NF-κB (3). IκBα binds to NF-κB at a position that blocks nuclear translocation. However, phosphorylation-dependent degradation of IκBα produces dissociation of NF-κB from IκBα, resulting in translocation of NF-κB to the nucleus (3). Therefore, to determine whether IκBα might be involved in regulating the age-related changes in NF-κB activation in the gastric mucosa, the levels of total and phosphorylated forms of IκBα were determined in isolated gastric mucosal cells by Western immunoblot. As shown in Fig. 7, levels of the total or phosphorylated form of IκBα in gastric mucosal cells were not affected by aging.

Fig. 6. Representative Western blots showing changes in p50 (A), p52 (B), and p65 levels (C) in gastric mucosal nuclei during aging. Aliquots of nuclear extracts (20 µg protein) from freshly isolated gastric mucosal cells from 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats were subjected to Western blot analysis. Relative changes in p50, p52, and p65 levels among the 3 age groups, as determined by densitometric analysis, are shown in the histogram.

Fig. 7. Representative Western blots showing changes in phosphorylated IκBα (A) and total IκBα levels (B) in gastric mucosal cells during aging. Aliquots of mucosal cell lysates from 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats were subjected to Western blot analysis. Relative changes in phosphorylated IκBα and total IκBα levels among the 3 age groups, as determined by densitometric analysis, are shown in the histograms.
Previously, we (46) demonstrated that the age-related rise in EGFR tyrosine kinase activity in the gastric mucosa is accompanied by a parallel increase in membrane-bound precursor forms of TGF-α. In view of this, we (37, 46) suggested that TGF-α might play a critical role in regulating EGFR tyrosine kinase through an autocrine/juxacrine mechanism. Results of our current investigation show that aging is also associated with increased transcriptional activity of AP-1 and NF-κB in the gastric mucosa. To determine whether conditions that activate EGFR will also augment the transcriptional activity of AP-1 and NF-κB during aging, we examined the effect of TGF-α on the DNA binding activity of AP-1 and NF-κB in gastric mucosal cells from young and aged rats. We have observed that exposure of freshly isolated gastric mucosal cells from aged rats to 1 nM TGF-α for 20 min markedly stimulates (50%) the DNA binding activity of both AP-1 and NF-κB over the corresponding controls (Fig. 8). In contrast, the same dose of TGF-α stimulated the transcriptional activity of only NF-κB in mucosal cells from young rats, compared with the corresponding controls (Fig. 8).

Because MAPKs play a key role in regulating the function of many transcription factors, including AP-1 and NF-κB, the next set of experiments was performed to examine the relationship between the activity of different MAPKs and AP-1 and NF-κB activation in the gastric mucosa during aging. Activity of ERKs, as assessed by the extent of phosphorylation of MBP by immunoprecipitated ERK1 and ERK2, revealed a progressive rise with advancing age (Fig. 9). The enzyme activity was found to be 55% and 95% higher in 12- to 14- and 22- to 24-mo-old rats, respectively, compared with their 4- to 6-mo-old counterparts (Fig. 9). In addition, activation of ERKs was also determined by examining the levels of phosphorylated ERKs by Western blot. Levels of phosphorylated ERKs in gastric mucosal cells from 22- to 24-mo-old rats were found to be ~60% higher than in their younger counterparts (Fig. 9). The relative concentration of ERKs (total) was also found to be ~30% higher in the gastric mucosa of 22- to 24-mo-old rats compared with young animals (Fig. 9).

In the next set of experiments, the age-related changes in JNK1 and p38 activities in the gastric mucosa were examined. Changes in JNK1 activity in the gastric mucosa during aging were very similar to what we have observed for ERKs. Activity of JNK1, as assessed by the extent of c-jun substrate phosphorylation by immunoprecipitated JNK1, was increased by 100% and 230% in 12- to 14- and 22- to 24-mo-old rats, respectively, compared with 4- to 6-mo-old animals (Fig. 10). In contrast, aging was associated with decreased activation of mucosal p38 MAPK activity, as evidenced by the decreased levels of phosphorylated p38 in 12- to 14- and 22- to 24-mo-old rats compared with 4- to 6-mo-old animals (Fig. 11). In 12- to 14- and 22- to 24-mo-old rats, p38 MAPK activity in the gastric mucosa was found to be about one-third of that observed in 4- to 6-mo-old animals (Fig. 11). On the other hand, no significant difference in total mucosal p38 levels was observed among different age groups (Fig. 11).

Finally, it should be stated that although a representative autoradiograph from one experiment was presented, each analysis was repeated three to four times.

![Fig. 8. Representative autoradiograph from EMSA analysis showing induction of AP-1 (A) and NF-κB binding activities (B) by transforming growth factor-α (TGF-α) in gastric mucosa during aging. Freshly isolated gastric mucosal cells from 4- to 6- and 22- to 24-mo-old Fischer 344 rats were incubated in the absence or presence of 1 nM TGF-α for 20 min. Aliquots of nuclear extracts (10 µg protein) from treated cells were subjected to EMSA analysis with 32P-labeled oligonucleotide probe containing consensus sequence of AP-1 or NF-κB. Relative changes in induction of AP-1 and NF-κB DNA binding activities by TGF-α among 2 age groups, as determined by densitometric analysis, are shown in the histograms.](http://ajpgi.physiology.org/)
with mucosal cells isolated from three rats in each age group.

**Discussion**

The structural and functional integrity of the gastrointestinal mucosa are maintained by the constant renewal of cells. Therefore, a detailed knowledge of mucosal cell proliferation and the regulation of this process is essential for a better understanding of the normal aging process as well as many gastrointestinal diseases (including malignancy) that arise from dysregulation of growth (25). Accumulating data (19, 27, 28, 30) suggest that gastrointestinal epithelial cells undergo age-dependent changes in their proliferative rate. Previously, we (38) demonstrated that in rats, gastric mucosal proliferative activity remains elevated during the first 2 wk of life and then decreases dramatically over the next 2–3 wk. Conversely, morphological and biochemical studies have demonstrated that in Fischer 344 rats, aging is associated with increased mucosal proliferative activity in the stomach (2, 37, 36), small intestine (2, 16), and large intestine (2, 17).

Although the intracellular mechanisms responsible for the age-related rise in gastrointestinal mucosal proliferative activity remain to be fully elucidated, results from earlier studies (46) suggest a role for EGFR in regulating this process. We (46) have demonstrated that the expression and tyrosine kinase activity of EGFR as well as levels of TGF-α are higher in the gastric mucosa of aged rats than in young rats. In addition, our recent preliminary data show that aging is also associated with increased sensitivity of gastric mucosal EGFR to EGF and TGF-α so that low doses of these ligands, which are ineffective in young rats, can activate EGFR tyrosine kinase in the gastric mucosa of aged animals (34).

Induction of intrinsic tyrosine kinase activity of EGFR triggers a complex array of enzymatic events through activation of Ras converging on MAPKs, which following translocation to the nucleus activate transcription factors (41, 42, 47). Our current observation that aging is associated with increased activation of ERKs and JNK1 as well as AP-1 and NF-κB suggests that induction of the EGFR signaling pathway is partly responsible for the age-related rise in gastric mucosal proliferative activity. This inference is further supported by the observation that TGF-α, one of the primary ligands of EGFR, causes further stimulation of NF-κB and AP-1 transcriptional activity in the gastric mucosa of aged rats.

Overexpression of c-erbB-2/neu, the structural and functional homologue of EGFR, which has been implicated in neoplastic transformation of cells (5, 8, 26, 50), has also been associated with increased activation of

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**Fig. 9.** Representative autoradiographs showing changes in extracellular signal-related kinase (ERK) activity (A), levels of phosphorylated ERK (B), and total ERK levels (C) in gastric mucosa during aging. Aliquots of gastric mucosal cell lysates from 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats were assayed for ERK activity using myelin basic protein (MBP) as a substrate (A), levels of phosphorylated ERK using antibodies against phosphorylated ERK1 and ERK2 (B), and levels of total ERK using antibodies against ERK1 and ERK2 (C). Relative changes in ERK activity and phospho-ERK and total ERK levels among the 3 age groups, as determined by densitometric analysis, are shown in the histogram.

**Fig. 10.** Representative autoradiograph showing changes in c-Jun NH2-terminal kinase 1 (JNK1) activity in gastric mucosa during aging. Aliquots of gastric mucosal cell lysate from 4- to 6-, 12- to 14-, and 22- to 24-mo-old Fischer 344 rats were assayed for J NK1 activity using c-Jun as a substrate. Relative changes in J NK1 activity among the 3 age groups, as determined by densitometric analysis, are shown in the histogram.
AP-1 and NF-κB (11). Previously, we (46) demonstrated that tyrosine kinase activity and mRNA levels of ErbB-2/neu are also considerably higher in the gastric mucosa of aged animals than young animals. Together, the results suggest that both EGFR and ErbB-2 are involved in modulating the transcriptional activity of AP-1 and NF-κB and in turn the proliferative activity in the gastric mucosa during aging.

Previous study (20) has demonstrated a relationship between activation of AP-1 and cell proliferation. However, the major components of the transcription factor AP-1 are encoded by two families of genes related to the protooncogenes c-fos and c-jun, the products of which associate to form a variety of homo- and heterodimers that bind to a common site, resulting in transcription of a number of genes involved in cell proliferation (45). Although several mechanisms are thought to be involved in inducing AP-1 activity, they can be broadly classified as those that stimulate the activity and those that increase the abundance of AP-1 (1). Our observation that the age-related rise in AP-1 activity, as evidenced by the increased binding of AP-1 to DNA, is associated with a concomitant rise in c-jun and c-Fos levels suggests that aging affects the mechanisms that regulate phosphorylation and expression of AP-1-family members in the gastric mucosa. Whether the increased mucosal levels of c-jun and c-Fos are the result of enhanced synthesis, however, remains to be determined.

Like AP-1, transcriptional activity of NF-κB is also augmented in the gastric mucosa of aged rats. This observation is similar to what has been noted in the liver (15). Although the regulatory mechanisms for the increase in mucosal NF-κB DNA binding activity remain to fully be elucidated, our observation that protein levels of p52, but not p50 and p65, in the nuclear fraction are increased in aged gastric mucosa suggests that high p52 levels could enhance the formation of the NF-κB complex with either p50 or p65. Additionally, alterations in phosphorylation of p50 and p65 could also play a role in modulating the binding activity of NF-κB in the gastric mucosa during aging. However, the age-related rise in activation of NF-κB in the gastric mucosa could not be attributed to decreased levels or increased degradation of IκBα, an intracellular protein that functions as a primary inhibitor of NF-κB (3). Our current data show that aging produces no significant change in either the total or phosphorylated form of IκBα. This observation is similar to what has been noted in a number of other tissues in aged rats (15).

The three MAPKs (ERKs, JNKs, and p38) respond to different stimuli. Whereas ERKs are primarily responsible for responding to proliferation signals, JNKs and p38 respond to cellular stress (10, 21, 23). Our current observation that with aging ERKs and JNK1, but not p38, are induced in the gastric mucosa suggests that all three MAPKs are not equally affected by aging. Because ERKs, which are known to be activated by several growth factors, including the EGF family of peptides (41), are linked to cell proliferation, it is reasonable to assume that induction of ERKs is an essential intracellular event in the EGFR signaling pathway for the age-related rise in proliferative activity in the gastric mucosa. Although JNKs and p38 MAPKs are thought to respond primarily to stress signals, gastric and intestinal injuries that induce activation of EGFR also activate ERKs and JNKs (13, 33, 41). Thus our current observation of the age-related rise in JNK1 activity in the gastric mucosa could be partly the consequence of increased activation of EGFR. Although the detailed EGFR signaling events leading to JNK1 activation remain to be determined, it is possible that the age-related activation of JNKs is mediated by Ras, a key factor in the EGFR signal transduction pathway (41). We believe it is unlikely that the age-related induction of JNK1 is due to stress because the related stress kinase p38 is not activated in the gastric mucosa of aged rats. In fact, the levels of phosphorylated p38 are found to be considerably lower in the gastric mucosa of aged rats than young rats.
could not be attributed to decreased protein levels of p38, which were not significantly different among the three age groups.

In conclusion, our data demonstrate that aging is associated with a marked induction of AP-1 and NF-κB transcriptional activity in the gastric mucosa, which could be further activated by TGF-α, one of the primary ligands of EGFR. Aging is also associated with increased activation of ERKs and JNK1, but not p38, suggesting a role for ERKs and JNK1 in regulating AP-1 and NF-κB transcriptional activity in the gastric mucosa during aging. These events may partly be responsible for the age-related rise in proliferative activity in the gastric mucosa.

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