Induced Autocrine Signaling through the Epidermal Growth Factor Receptor Contributes to the Response of Mammary Epithelial Cells to Tumor Necrosis Factor α*

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In contrast to the well known cytotoxic effects of tumor necrosis factor (TNF)α in many mammary cancer cells, we have found that TNF stimulates the proliferation and motility of human mammary epithelial cells (HMECs). Since the response of HMECs to TNF is similar to effects mediated by epidermal growth factor receptor (EGFR) activation, we explored the potential role of cross-talk through the EGFR signaling pathways in mediating cellular responses to TNF. Using a microarray enzyme-linked immunoassay, we found that exposure to TNF stimulated the dose-dependent shedding of the EGFR ligand transforming growth factor α (TGFα). Both proliferation and motility of HMECs induced by TNF was prevented either by inhibiting membrane protein shedding with a metalloprotease inhibitor, by blocking epidermal growth factor receptor (EGFR) kinase activity, or by limiting ligand-receptor interactions with an antagonistic anti-EGFR antibody. EGFR activity was also necessary for TNF-induced release of matrix metalloprotease-9, thought to be an essential regulator of mammary cell migration. The cellular response to TNF was associated with a biphasic temporal pattern of extracellular signal-regulated kinase (ERK) phosphorylation, which was EGFR-dependent and modulated by inhibition of metalloprotease-mediated shedding. Significantly, the late phase of ERK phosphorylation, detectable within 4 h after exposure, was blocked by the metalloprotease inhibitor batimastat, indicating that autocrine signaling through ligand shedding was responsible for this secondary wave of ERK activity. Our results indicate a novel and important role for metalloprotease activation and EGFR transmodulation in mediating the cellular response to TNF.

Tumor necrosis factor (TNF)α is a potent cytokine produced by many cell types in response to inflammation, infection, and environmental stress. Originally discovered for its ability to induce hemorrhagic necrosis in tumor cells (1), TNF is perhaps best known for inducing cytotoxicity and apoptosis in transformed cells. In many non-transformed cells, however, TNF is thought to mediate an important prosurvival role. For example, cell death in response to TNF is rarely observed in normal cells unless inhibitors of transcription or translation are concurrently administered, suggesting gene regulatory pathways regulated by TNF signaling include cytoprotective pathways (2, 3). In particular, normal mammary epithelial cells (MECs) are relatively resistant to TNF cytotoxicity as compared with mammary cancer cells (4, 5), and some reports suggest TNF plays a physiological role as both a survival factor and mitogen in normal mammary epithelium (6–8). For example, in primary rat MECs, TNF stimulates proliferation and up-regulates matrix metalloproteases necessary for cell motility and branching morphogenesis (6, 7). MEC proliferation and branching during puberty is also delayed in TNF null mice (9). Consistent with having an important physiological role, expression of TNF and its receptors is tightly regulated throughout mammary development and is elevated during pregnancy prior to the onset of lactation and mammary proliferation (8). Despite these reports, it is not clear whether these are direct TNF signaling responses or secondary responses mediated through interactions with additional growth factor pathways involved in MEC regulation.

The cellular effects of TNF are initiated by two distinct surface receptors (TNFR1 and TNFR2), which are expressed on most cell types. Numerous post-receptor signaling pathways appear to be activated by TNF, and this might explain the diversity of cellular responses elicited. However, this complexity has also made it challenging to identify key events involved in the induction of specific cellular responses. For instance, all of the major mitogen-activated protein kinase (MAPK) pathways (p38, JNK, and ERK families) can be stimulated by TNF, although the signaling intermediates involved are still poorly understood (10). These pathways converge on key transcription factors such as NFκB and AP-1, providing a mechanism for integration at the level of gene regulation (11). Additional prosurvival pathways independent of NFκB, such as Akt activation, also contribute to the anti-apoptotic effects of TNF in some systems (12, 13). This signaling complexity is thought to provide a flexible mechanism that can elicit a range of cellular

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The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; MEC, mammary epithelial cell; MAPK, mitogen-activate protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; HMEC, human mammary epithelial cell; MMP, matrix metalloprotease; mAb, monoclonal antibody; TGF, transforming growth factor; ELISA, enzyme-linked immunosorbent assay; RANTES, regulated on activation normal T cell expressed and secreted; PDGF, platelet-derived growth factor.
responses to different extracellular environments where elevated levels of soluble TNF are present. Identifying the postreceptor binding events that underlie this complexity is therefore an important goal. 

There is growing appreciation for the importance of crosstalk among heterologous receptor signaling pathways in coordinating cellular responses. A potentially important pathway for modulating TNF-mediated responses is that of the epidermal growth factor receptor (EGFR). Earlier studies have reported relationships between TNF and EGFR signaling cascades at several levels of regulation. In cervical and pancreatic cancer cell lines, TNF stimulates expression of ligands for EGFR (14, 15). A study in fibroblasts reported that transactivation of EGFR by TNF plays a pivotal role in NFκB activation (16). TNF also sensitizes carcinoma cell xenografts to anti-EGFR therapy, indicating that TNF and EGFR signaling pathways in cancer cells are interconnected (17). The importance of EGFR signaling in regulation of normal mammary epithelial cell proliferation, motility, and survival is well established (18–21). We were, therefore, interested in investigating the effects of TNF in normal human mammary epithelial cells (HMECs) and determining whether the effects involved EGFR signaling. Our studies revealed that low levels of TNF stimulate proliferation and cell motility through mechanisms that are dependent, at least in part, on EGFR autocrine signaling. The results also indicate that the cellular response to TNF is strongly influenced by its ability to modulate other receptor systems by inducing shedding of growth factors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Human mammary epithelial cells (HMECs), strain 184A1 (22), were routinely cultured in DFCI-1 medium supplemented with 12.5 ng/ml human epidermal growth factor (Calbiochem) as previously described (20). Batimastat was generously provided by British Biotech Pharmaceuticals Ltd. Recombinant soluble human TNF, the EGFR kinase inhibitor PD153035, and MMP-3 Inhibitor I were purchased from Calbiochem. The EGFR-neutralizing monoclonal antibody 225 (225 mAb) was purified from hybridoma supernatant (10–H9262) with 10 g/ml of collagen type I (10–H9262) and 2-liter ELISA assays on the microarray was developed individually, and assay conditions were optimized based on reported concentrations of that particular marker in serum. To obtain quantitative measures of each protein, a series of standards was analyzed in parallel to the analysis of cell culture medium. This was undertaken by preparing a series of cell lysates that were serially diluted 2-fold to generate a set of seven standards. Standards were prepared in the same solution as used for the culture medium samples (see below). Each dilution of the standard mixture was analyzed in duplicate using individual arrays on different slides. Prior to analysis, cell culture supernatants were diluted 2-fold in blocking buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% blocking reagent (PerkinElmer Life Sciences). Although the assay provided quantitative data for 16 proteins, the concentrations of 5 proteins in cell culture medium were outside the range of the series of standards. For each treatment, culture medium from three dishes was collected, and each sample was analyzed on two arrays that were printed on separate glass slides.

**Gelatin Zymography—**The presence of secreted MMP-2 and MMP-9 in HMEC-conditioned medium was analyzed by gelatin zymography (24). HMECs were exposed to TNF, with or without additional inhibitors in serum-free and EGF-free medium. After 24 h, conditioned medium was collected and concentrated by centrifugal filtration (Millipore), and proteins were separated by SDS-PAGE. The volumes loaded were normalized to account for differences in cell number. After electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h at 37 °C, followed by 24 h in Tris-HCl buffer containing 0.2 mM NaCl and 5 mM CaCl2 (pH 7.5). After staining with Coomassie Blue, the gels were cleared, and images obtained using a chemiluminescent imager in bright field transmission mode.

**Blot and Immunoprecipitation—**Total or active MAPK proteins were measured by immunoblot analysis using polyclonal antibodies that recognize total p44 (ERK1) and p42 (ERK2) MAPK or dually phosphorylated specific MAPK protein (Promega) as previously described (25). Secreted MMP-9 protein levels were measured by Western blot analysis of HMEC-conditioned medium using procedures described for gelatin zymography, and detection was accomplished with a mouse monoclonal anti-MMP-9 antibody (R&D Systems) and a chemiluminescent imager (LumiImager, Roche Applied Science). For analysis of EGFR phosphorylation, total EGFR was immunoprecipitated by incubation of whole cell lysates (200 μg) with 225 mAbs conjugated to streptavidin beads for 15 min at 4 °C, and the immunocomplexes were washed using an avidin column. After gently eluting the protein was eluted in buffer containing 100 mM ammonium bicarbonate and 8 M urea, and equivalent amounts of protein were analyzed for phosphorylation status by Western blot using an anti-phosphotyrosine antibody (Upstate Biotechnology Inc.) and chemiluminescence detection.

**RESULTS**

**TNF-stimulated HMEC Growth and Motility Involves EGFR—**It has previously been reported that, unlike mammary cancer cells, non-transformed mammary cells are resistant to the cytotoxic effects of TNF (4, 5). Consistent with this previous work, we found that low concentrations of TNF were not only non-cytotoxic to HMECs but actually stimulated cell survival and growth after several days in minimal medium (Fig. 1A). In the absence of EGF or additional added growth factors, TNF concentrations as low as 0.1 ng/ml caused a quantifiable increase in cell density over 3 days in culture as compared with medium lacking TNF. Routinely, we found that maximal stimulation of HMEC growth was achieved at concentrations ranging between 5 and 10 ng/ml TNF. At these low concentrations of cytokine, no evidence for induction of apoptosis was observed, as evaluated by nuclear morphology and trypan blue exclusion tests (results not shown).

**To investigate the potential role of EGFR signaling in the growth stimulatory effects of TNF, cell growth assays were conducted in the presence and absence of a potent and specific
sine kinases requires micromolar or higher concentrations (26). PD153035 used in these experiments blocked phosphorylation of EGFR induced by EGF treatment (results not shown and Fig. 4). The results shown in Fig. 1B demonstrate that cell numbers were increased over 2-fold by 5 ng/ml TNF treatment, an increase comparable to that achieved by treatment with EGF (1 ng/ml). In the presence of the EGFR kinase inhibitor, however, TNF was ineffective and resulted in cell densities similar to untreated control cultures. As expected, a similar suppression of EGF-induced growth was achieved with PD153035 (Fig. 1B). In addition, TNF-induced cell growth was inhibited by co-treatment with a neutralizing antibody (225 mAb) (Fig. 1B), which blocks EGFR ligand binding (27), suggesting that, even in the absence of added EGFR ligands, the apparent proliferative effects of TNF in HMECs are dependent on EGFR signaling.

HMECs constitutively produce EGFR ligands that, when released into the extracellular environment by regulated proteolysis, stimulate the receptor in an autocrine manner. A tight correlation between the proteolytic release of EGFR ligands and growth of HMECs has been demonstrated (28, 29). In particular, the hydroxamate-based metalloprotease inhibitor, batimastat, has been shown to reduce HMEC mitogenesis in direct proportion to its ability to inhibit endogenous TGFα release (28). Thus, we reasoned that if the mechanism of cross-talk between TNF and EGFR requires proteolytic release of EGFR ligands, batimastat should block the ability of TNF to stimulate HMEC growth. In support of this hypothesis, we found that treatment with batimastat inhibited TNF-induced growth in a dose-dependent manner (Fig. 1C). Relatively low concentrations of batimastat (0.1 μM) were sufficient to inhibit TNF-induced HMEC growth, demonstrating that the growth stimulatory effects of exogenous TNF are dependent on metalloprotease activity. Interestingly, with increasing concentrations of batimastat, cell growth could be reduced below untreated control levels, suggesting that in addition to TNF-inducible pathways, constitutive metalloprotease-dependent pathways are active in HMECs and contribute to basal cell growth rates.

In the absence of exogenous growth factors, HMECs grow as compact colonies of cells, suggesting a low level of migratory behavior (Fig. 2). During our initial experiments we observed a scattering effect in HMEC cultures exposed to low levels of TNF, suggesting that this cytokine might also stimulate HMEC motility. To determine whether TNF-induced cell motility involved EGFR signaling, we investigated the effect of co-treatment with EGFR-neutralizing antibody on the pattern of cell growth in the presence and absence of TNF. As shown in Fig. 4A, cell scattering induced by TNF was eliminated when 225 mAb was included as a co-treatment. In contrast, the increase in cell motility induced by TNF was not affected by addition of an unrelated IgG control antibody, indicating the effects observed specifically involve EGFR (Fig. 2A). Additional experiments showed that, like 225 mAb, co-treatment with PD153035 suppressed cell scattering by TNF (Fig. 2B). We also investigated whether TNF-induced cell motility was dependent on metalloprotease activity, as it was in the case of cell growth. As shown in Fig. 2B, TNF increased cell scattering, but co-treatment with batimastat resulted in the same compact colony-like pattern of growth observed in the absence of TNF.

To evaluate the cell migration response quantitatively, a modified Boyden chamber assay was used to measure the fraction of cells that migrated across a collagen matrix in response to TNF or co-treatments. As is summarized in Fig. 3, TNF treatment (4 h) increased the number of cells that migrated across a collagen matrix 2-fold compared with untreated controls (Fig. 3). Co-treatment with batimastat completely inhibi-
Fig. 2. Stimulation of HMEC motility by TNF is EGFR- and metalloprotease-dependent. A, representative phase contrast images (×400) of HMECs cultured in uncoated plastic dishes for 24 h in the presence of no added growth factors (top left), 5 ng/ml TNF (top right), TNF with 10 μg/ml 225 mAb (bottom left), or TNF with a nonspecific IgG control antibody (bottom right). B, DIC images (×200) of HMECs cultured in the absence of added growth factors (top left), 5 ng/ml TNF (top right), TNF combined with the EGFR kinase inhibitor PD153035 (bottom left), or TNF combined with batimastat (bottom right). The images shown are representative of experiments that were repeated twice with similar results.

Fig. 3. TNF-induced migration of HMECs through collagen matrix is EGFR- and metalloprotease-dependent. HMECs were seeded over collagen-coated Transwell chambers in EGF-free medium, and after 4-h treatment with TNF (5 ng/ml), batimastat (10 μM), PD153035 (100 nM), or combined TNF plus inhibitors, the fraction of cells that migrated through the collagen matrix was determined by staining the cells with SyberGreen and analyzing cell number using a fluorescent plate reader (see “Experimental Procedures”). Values for PD or PD plus TNF treatments are the mean of duplicate measurements (duplicate chambers). All other values shown are the mean ± S.D. from triplicate measurements, normalized to untreated controls. The results shown are representative of experiments that were repeated twice with similar results.

The results most relevant to this study is shown in Fig. 5. The cancer marker CA15-3 (also known as MUC1 mucin) and RANTES displayed an increased net release after TNF treatment.
These results are consistent with previous studies showing TNF regulates the production and release of these proteins in other epithelial cell types (30, 31). TNF-induced release of CA15-3 and RANTES was unaffected by the presence of 225 mAb, indicating this response was independent of EGFR signaling. In contrast, extracellular levels of the metalloprotease MMP-9 and platelet-derived growth factor (A-chain) were increased by TNF treatment, but addition of 225 mAb significantly reduced the release of these proteins. In the case of TGFα production, an endogenous ligand for EGFR, co-treatment with 225 mAb significantly enhanced its level, probably by preventing its uptake and degradation by cells (29). The release of other marker proteins, including PDGF-B (Fig. 5), basic fibroblast growth factor, HER-2, sICAM-1, and hepatocyte growth factor (results not shown) was not significantly affected by any treatment, indicating that the TNF-mediated responses as well as the ability of 225 mAb to modulate these responses were specific to a subclass of released proteins.

The microarray ELISA provided a rapid means of screening potential proteins shed or secreted in response to TNF in HMECs. Of particular interest are those proteins whose extracellular release, in collaboration with EGFR signaling pathways, could contribute to the migratory phenotype induced by TNF. Therefore, additional experiments focused on using independent methods to verify the microarray ELISA results obtained for TGFα and MMP-9. Among the EGFR ligands expressed in HMECs, TGFα has been shown to signal through autocrine mechanisms (28, 29). Autocrine presentation of EGFR ligands is thought to provide a spatially restricted signal that directs HMEC migration (32). Using an independent commercial enzyme-linked immunosorbent assay to measure levels of soluble TGFα in HMEC-conditioned medium, we could not detect an increase in TGFα levels over control levels following treatment with TNF alone (Fig. 6). However, when the 225 mAb was added to prevent EGFR utilization of the ligand, levels of extracellular TGFα accumulated with increasing concentrations of TNF (Fig. 6). These results not only confirmed the microarray ELISA results but clearly demonstrated that shedding of TGFα occurs in a TNF concentration-dependent manner.

Activation and release of MMP-9 is thought to play an important role in mammary cancer and invasion (33). To further investigate the potential regulatory role of EGFR in TNF-stimulated release of this metalloprotease, gelatin zymography was used to measure MMP-9 activity in conditioned medium from HMECs. The results shown in Fig. 7 confirmed TNF significantly increased MMP-9 activity. Importantly, co-treatment with the EGFR kinase inhibitor completely suppressed TNF-induced MMP-9 activity (Fig. 7A). In contrast, MMP-2 activity was found to be constitutive in HMEC-conditioned medium and not altered by either TNF or the EGFR kinase inhibitor, indicating the effects on MMP-9 production are selective. Western blot analysis further confirmed that extracellular MMP-9 protein levels were dramatically increased by TNF treatment (Fig. 7B). Inhibition of TNF-induced MMP-9 levels was also observed following co-treatment with the neutralizing 225 mAb (Fig. 7B). The induction of MMP-9 secretion by TNF was also concentration-dependent and was observed at the same low concentrations of TNF that stimulate cell migration (Fig. 7C). Thus, several independent approaches demonstrate that the activation and release of MMP-9 from HMECs by TNF is dependent on EGFR activity.
Conventional views of signal transduction involve highly specific interactions of growth factor receptors with distinct ligands, which in turn results in rapid activation of a limited but specific set of signaling cascades that ultimately dictate the temporal pattern of ERK activation.

Dependence of ERK activation on EGFR activity was examined by treating HMECs with a panel of inhibitors at various time points after TNF exposure. Western blot analysis revealed that the level of ERK phosphorylation in response to TNF plus U0126, a selective inhibitor of ERK activation, was significantly inhibited compared to TNF alone. This inhibition was concentration-dependent, indicating that ERK activation is partially dependent on EGFR activity.

To determine whether EGFR transactivation drives either the primary or secondary phase of ERK activity, experiments were conducted to determine if co-treatment with PD153035 altered TNF-induced ERK phosphorylation. Interestingly, the results demonstrated that co-treatment with PD153035, at concentrations which block EGFR activation (Fig. 4A), significantly inhibited TNF-induced ERK phosphorylation at all time points examined (Fig. 4B). Similar to previous experiments, TNF alone stimulated a rapid phase of ERK activation followed by a secondary increase beginning at 4 h. In the presence of PD153035, the level of ERK phosphorylation in response to TNF remained at or below control levels at all time points. Importantly, the effect of PD153035 was not due to a general suppression of ERK activation. For example, in contrast to effects observed with TNF treatment, ERK phosphorylation in response to hepatocyte growth factor (HGF) for 15 min, in either the presence or absence of EGFR inhibitor, and ERK phosphorylation was measured as described above.

The importance of ERK phosphorylation for TNF-induced cell growth was further evaluated using a direct and selective inhibitor of ERK activation. As shown in Fig. 4B, co-treatment of HMECs with U0126, a selective inhibitor of ERK activation, blocked both basal and TNF-induced cell growth. As a control, an inhibitor of the matrix metalloprotease MMP-3 was included, based on a previous study that demonstrated this inhibitor did not interfere with the release of EGFR ligands from HMECs (28). Consistent with this earlier report and in contrast to the results obtained with batimastat, the MMP-3 inhibitor had no effect on TNF-induced cell growth. Furthermore, this inhibitor did not alter the level of ERK phosphorylation induced by TNF (Fig. 4A).

**DISCUSSION**

The metalloprotease inhibitor batimastat modulates the temporal pattern of TNF-induced ERK phosphorylation. A, HMECs were grown to ~90% confluence in complete medium, and then medium was switched to EGF-free medium for 16 h. The cells were treated with TNF (10 ng/ml) in the presence or absence of batimastat (10 μm) for the times indicated. An antibody that selectively recognizes dual phosphorylated ERK2 (upper and middle blots) was used to measure ERK activity by Western blot. As is shown in the lower blot in A, Western blot analysis with an antibody that recognizes total ERK protein demonstrated ERK protein levels remained unchanged throughout the treatment times. B, HMECs were treated with TNF in the presence or absence of the EGFR kinase inhibitor for the times indicated, and either phosphorylated ERK (upper blot) or total ERK were measured by Western blot. C, HMECs were treated with 5 ng/ml hepatocyte growth factor (HGF) for 15 min, in either the presence or absence of EGFR inhibitor, and ERK phosphorylation was measured as described above.

**Fig. 8.** The metalloprotease inhibitor batimastat modulates the temporal pattern of TNF-induced ERK phosphorylation. A, HMECs were grown to ~90% confluence in complete medium, and then medium was switched to EGF-free medium for 16 h. The cells were treated with TNF (10 ng/ml) in the presence or absence of batimastat (10 μm) for the times indicated. An antibody that selectively recognizes dual phosphorylated ERK2 (upper and middle blots) was used to measure ERK activity by Western blot. As is shown in the lower blot in A, Western blot analysis with an antibody that recognizes total ERK protein demonstrated ERK protein levels remained unchanged throughout the treatment times. B, HMECs were treated with TNF in the presence or absence of the EGFR kinase inhibitor for the times indicated, and either phosphorylated ERK (upper blot) or total ERK were measured by Western blot. C, HMECs were treated with 5 ng/ml hepatocyte growth factor (HGF) for 15 min, in either the presence or absence of EGFR inhibitor, and ERK phosphorylation was measured as described above.

**Fig. 7.** Secretion of MMP-9 by TNF is co-regulated by EGFR. A, HMECs were left untreated or exposed to TNF (5 ng/ml) for 24 h. Conditioned medium was concentrated using Centricon filtration cartridges, and volumes normalized to cell number were used to measure MMP-9 activity by gelatin zymography, as described under "Materials and Methods." B, HMECs were left untreated, treated with TNF, 225 μg/ml (10 μg/ml), or combined TNF plus 225 μg/ml for 24 h. Conditioned medium was harvested as described above and analyzed for MMP-9 protein content by Western blot analysis. C, dose-response relationship for MMP-9 induction by TNF as measured by gelatin zymography (24 h after treatment).

**Temporal Aspects of MAPK Activation**—Depending on the cell type and context, a variety of signaling pathways are stimulated by TNF, including the three major families of the MAPK cascades. Of particular importance are the extracellular signal-regulated kinases (ERKs), which have been reported to regulate mammary cell motility and invasion and stimulate metalloproteases involved in EGFR ligand release (34, 35). Prolonged activation of ERK has also been reported to be necessary for stimulation of MMP-9 activity (36). To determine whether ERK is activated in HMECs by TNF, we conducted Western blot analysis using an antibody that selectively recognizes dual-phosphorylated and active ERK2. Interestingly, the results shown in Fig. 8A revealed that TNF stimulates a biphasic temporal pattern of ERK activation, with a rapid robust activation peaking at ~15 min, followed by a second wave of ERK activation beginning between 3 and 4 h and progressing up to at least 8 h. Based on the findings that TNF stimulates the release of ligands, which may signal through autocrine mechanisms, we hypothesized that the second wave of ERK phosphorylation may be a reflection of the slower activation of a diverse range of autocrine factors whose signaling converge on ERK. To investigate this possibility, we determined whether the temporal pattern of ERK activation was altered when batimastat was used to broadly inhibit metalloprotease activity and ligand shedding. Significantly, addition of batimastat with TNF reduced both the primary and secondary waves of ERK activation (Fig. 8A, middle panel). However, the major effect was observed on the second wave of TNF-induced ERK activity, which was nearly eliminated. This indicates that the biphasic pattern of ERK phosphorylation is highly dependent on metalloprotease activity. Western blots conducted using an antibody that recognized total ERK1 and ERK2 proteins demonstrated that the levels of these proteins were not altered during the time course of TNF exposure (Fig. 8A, bottom panel). Thus, temporal aspects of TNF-induced signaling appear to be significantly modulated by metalloprotease activity.

To determine whether EGFR transactivation drives either the primary or secondary phase of ERK activity, experiments were conducted to determine if co-treatment with PD153035 altered TNF-induced ERK phosphorylation. Interestingly, the results demonstrated that co-treatment with PD153035, at
The levels of TGFβ1 presence or absence of a selective inhibitor of ERK activation (U0126; HMECs were treated with TNF (5 ng/ml) in the presence of a selective inhibitor of ERK activation (U0126; 10 μM), or the MMP-3 inhibitor I (5 μM). Cell density was determined after 3 days treatment as described for experiments in Fig. 1. The Western blot results shown in A demonstrate that co-treatment with U0126, but not MMP-3 inhibitor, inhibited TNF-induced ERK phosphorylation. Results (mean ± S.D.) of triplicate cell density measurements from each of two independent treatments are shown in B.

Inhibition of ERK activation blocks TNF-stimulated HMEC growth. HMECs were treated with TNF (5 ng/ml) in the presence or absence of a selective inhibitor of ERK activation (U0126; 10 μM), or the MMP-3 inhibitor I (5 μM). Cell density was determined after 3 days treatment as described for experiments in Fig. 1. The Western blot results shown in A demonstrate that co-treatment with U0126, but not MMP-3 inhibitor, inhibited TNF-induced ERK phosphorylation. Results (mean ± S.D.) of triplicate cell density measurements from each of two independent treatments are shown in B.

In addition to modulating phenotypic responses to TNF by inhibiting EGFR, independent assays (Figs. 5 and 6) directly confirmed that treatment with TNF stimulated the release of TGFβ1 and resulted in stimulation of EGFR phosphorylation. The levels of TGFβ1 shedding induced by TNF were in accordance with previous measurements of TGFβ1 release in HMECs by separate approaches (29). Interestingly, the levels of TGFβ1 that accumulated in HMEC-conditioned medium remained low unless a neutralizing EGFR antibody was included. This implies that, upon stimulation of shedding by TNF, most soluble TGFβ1 is rapidly sequestered by EGFR, resulting in autocrine signaling. It has been shown that low levels of shed EGFR ligand are sufficient to drive autocrine responses and that, in HMECs, localized EGFR autocrine signaling is involved in directed cell migration (29, 32). Additional EGFR autocrine ligands not measured in this study, such as amphiregulin or heparin-binding EGF, could potentially be shed in response to TNF and perhaps contribute to the overall cellular response. Previous studies showing TNF-stimulated amphiregulin expression and autocrine signaling in cervical cancer cells (14) supports the idea that multiple autocrine ligands may contribute to the cellular response to TNF and that the levels of TGFβ1 shedding observed in this study may underestimate the overall magnitude of the induced autocrine activity.

Our results support the hypothesis that transactivation of EGFR signaling by TNF is the result of activation of the proteases involved in shedding of EGFR ligands. The proteolytic release of both TNF and EGFR ligands are stimulated by a number of pharmacological agents, including phorbol esters and calcium ionophores, suggesting common regulatory pathways are involved (35, 39, 40, 47, 48). Among the enzymes involved in regulated ligand shedding, TNF-converting enzyme (also known as ADAM-17C, O.) has been shown to mediate cleavage of both pro-TNF and pro-TGFβ (40–42). It has been reported that exposure of mammary cancer cells to TNF treatment stimulates the synthesis and release of soluble TNF and that this is associated with resistance to TNF cytotoxicity (43). The shedding of the p75 TNF receptor (TNFR2), which is also mediated by TNF-converting enzyme, is stimulated by TNF as well (44, 45). These reports imply soluble TNF promotes a positive feedback mechanism by activating the release of proteins processed by commonly regulated metalloprotease(s) and raise the interesting question of whether activation of metalloproteases involved in shedding might serve as a mechanism for coordinating signaling among divergent pathways.

Cross-talk between TNF and EGF receptor pathways could also have important developmental significance as well as therapeutic relevance. Most notably, the results of this study indicate that the metalloprotease MMP-9 (gelatinase B), a key protease involved in degradation of collagen and remodeling of the extracellular matrix, is induced by TNF in an EGFR-dependent manner (Fig. 7). Both the pharmacological agent PD153035 and 225 mAb suppressed the level of MMP-9 induction by TNF. Furthermore, this interaction was specific for MMP-9, because constitutive levels of MMP-2 were unaffected by either treatment (Fig. 6). The induction of MMP-9 is likely to be physiologically important, because it has been shown that, in the case of rat MECs, peptide inhibitors of MMP-9 disrupt...
TNF-stimulated growth and three-dimensional organization on reconstituted basement membrane (46). In HMECs, inhibition of EGFR autocrine signaling by 225 mAb also disrupts cellular organization on a reconstituted extracellular matrix (20). Because both MMP-9 expression and EGFR signaling are commonly increased in mammary tumors, it will be important in future studies to investigate whether the expression of the proteins is coordinately regulated in cancer and whether inflammatory mediators like TNF are involved.

An additional important contribution of this work is the demonstration that the induction of metalloprotease activity by TNF significantly influences the temporal pattern of MAPK activation. Treatment of HMECs with TNF resulted in two phases of ERK phosphorylation, with an initial rapid transient peaking at around 15 min followed by a slower increase in activation beginning between 3 and 4 h and progressing up to at least 8 h. The initial transient activation of ERK is likely due to rapid signaling events directly coupled with TNF receptors. Interestingly, the first phase of ERK phosphorylation was also inhibited by co-treatment with an EGFR kinase inhibitor, indicating even this rapid signaling event is dependent on EGFR (Fig. 8). The initial phase of ERK phosphorylation was partially reduced by batimastat, consistent with a role for metalloprotease-dependent activation of EGFR. Because batimastat only partially blocked the initial phase of ERK activation, it is not yet clear whether additional batimastat-insensitive metalloproteases are involved in EGFR transactivation, or whether ligand-independent mechanisms also contribute to the rapid cross-talk between TNF and EGFR pathways. The late phase of ERK activation, however, was eliminated by batimastat, consistent with the induction of metalloprotease activity by TNF as a primary mechanism responsible for this second wave of ERK activation. This latter phase likely reflects ERK activation mediated by a broad range of autocrine signaling pathways induced by TNF, in addition to EGFR.

As depicted in Fig. 10, our results suggest that by triggering metalloprotease-mediated release of ligands, TNF exposure results in a positive feedback loop of autocrine signaling that could modulate the timing and duration of signaling through heterologous receptor pathways that converge on ERK. The duration of ERK activation has been shown to be an important determinant in mammary cell responses to growth factors. For instance, the motility of mammary carcinoma cells in response to TGFβ is modulated by the duration and magnitude of ERK activity (34, 49). In many mammary carcinoma cell models, cell motility in response to TGFβ correlates with transient ERK activation, whereas in vitro invasion is associated with sustained activation of ERK (34). Induction of metalloproteases, which degrade or alter the extracellular matrix, including MMP-9, are thought to be key events in this invasion and migration. Although both TNF and ligands for the EGFR are known inducers of MMP-9 in mammary cells (46, 50), our results are the first to demonstrate that the induction of MMP-9 by TNF is dependent on EGFR signaling. We found that increases in MMP-9 activity after treatment of HMECs with TNF are not detectable until more than 8 h after exposure (data not shown), following the second prolonged wave of ERK activity. This temporal sequence of events is consistent with a previous report that showed prolonged ERK activation is necessary, although not sufficient for induction of MMP-9 by TNF (36). Interestingly, in models of keratinocyte migration, it was shown that ligands that are mitogenic, but fail to induce MMP-9 or cause colony dispersion, only transiently activate ERK, whereas colony dispersion and MMP-9 induction is associated with ligands that produce sustained ERK activity (49).

The results of our study are also consistent with recent mathematical modeling predictions that metalloprotease-dependent release of growth factor ligands can modulate the magnitude and duration of MAPK activity (51). In this sense, the critical events in TNF signaling are not restricted to the initial signaling cascades activated, but also result from positive feedback that occurs over hours (Fig. 8). Positive feedback induced by triggering autocrine signaling pathways has been predicted to be an effective mechanism for regulating cell behavior in a manner that is highly dependent on the context of the extracellular environment and the proximal receptor content of neighboring cells would influence the quantity and types of ligands which could bind back to the cell surface. Future studies that identify the regulatory proteins released in response to TNF in normal cells should lead to a more predictive understanding of key interactions between epithelial cells and the mammary stromal environment and ultimately identify unique approaches for disrupting these complex networks for therapeutic gain.

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