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13. ABSTRACT *(Maximum 200 words)*

We hypothesize that alterations in the regulation of growth by growth factors and cAMP during mammary tumor progression are related to MAP kinase signaling pathways known to be affected by cAMP and pertussis toxin (PT)-sensitive G proteins. Mammary epithelial cells from normal mouse mammary glands were compared to pregnancy-dependent (PDT) and ovarian-independent (OIT) mouse mammary tumors in serum-free, collagen gel cell culture. Previous studies suggested that the ERK cascade is only permissive for proliferation, and cAMP and hormones stimulate proliferation via nonERK PT-sensitive pathways. The possible involvement of superphysiological insulin/IGF-I receptor in PT sensitivity was examined. PT-sensitivity of proliferation and ERK activation is observed in the absence of insulin. However, in the presence of insulin, cAMP enhanced PT inhibition of ERK activity suggesting a PT-sensitive interaction of insulin/IGF-I pathways with cAMP. Examination of other pathways showed that neither the activation or inhibition of the p38 MAP kinase or changes in raf level correlated with stimulatory or inhibitory effects of cAMP on growth. Lysophosphatidic acid (LPA), which affects normal and tumor growth similarly to cAMP, was tested and found to activate MAP kinases (ERK, JNK, p38) in normal and OIT. LPA stimulation of JNK and ERK activity but inhibition of growth in OIT emphasizes that inhibition of proliferation in these tumors is does not correlate with MAP kinase inhibition and suggests that the previous observation of JNK inhibition by cAMP in OIT is not relevant to its negative effect on growth.

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Abbreviations

cAMP, 3'-5' cyclic adenosine monophosphate
C56, Compound 56 (EGF receptor phosphorylation inhibitor)
CREB, cAMP response element binding protein
EGF, epidermal growth factor
ERK, extracellular regulated mitogen-activated protein kinase (MAP kinase)
ECL, enhanced chemiluminescence
IGF-I, insulin-like growth factor I
JNK, c-jun protein kinase
LPA, 1-oleoyl-lysophosphatidic acid
MEC, mammary epithelial cell
MEK, MAP kinase kinase
OIT, ovarian-independent mammary tumor
PD, PD098059 (MEK inhibitor)
PDT, pregnancy-dependent mammary tumor
PKA, protein kinase A
PT, pertussis toxin

Introduction

The purpose of this research is to identify the mechanisms underlying the change in the proliferative response to 3'-5' cyclic adenosine monophosphate (cAMP) that we have previously observed during mammary tumor progression in our rodent systems (1). cAMP is a potent mitogen for normal mammary gland epithelium, weakly mitogenic for hormone-dependent mammary tumors and growth inhibitory to hormone-independent mammary tumors. cAMP-stimulated proliferation was inhibited by pertussis toxin (PT) in normal mammary epithelium but neither the inhibitory effect of cAMP nor basal cAMP-independent proliferation of hormone-independent mammary tumors was affected by PT (2). PT is a bacterial toxin that ADP-ribosylates $G\alpha i$ subunits and blocks activation of receptor-coupled heterotrimeric $G\alpha i\beta\gamma$ proteins. These findings indicate that through postreceptor crosstalk, pertussis toxin-sensitive $G\alpha i\beta\gamma$ pathways modulate cAMP-mediated proliferation.

These preliminary results led to the hypothesis that a critical alteration in growth regulation related to signaling pathways affected by cAMP and pertussis toxin-sensitive G proteins occurs during transformation and progression of mammary tumors from hormone-dependent to hormone-independent growth. An *in vitro* approach has been followed, taking advantage of our serum-free, primary cell culture system, to examine intracellular kinase pathways that may be altered during progression from normal mammary epithelium to hormone-independent mammary tumors.

Previously we reported that cAMP inhibited the activity of the mitogen activated protein kinase, ERK, in normal mammary epithelium, but did not affect ERK activity in pregnancy-dependent (PDT) or ovarian-independent (OIT) mammary tumors. Thus, the mitogenic effect of cAMP could not be related to a concurrent stimulation of ERK activity. However, the growth-stimulatory effect of cAMP and prolactin on normal and PDT cells in vitro was inhibited by the specific MEK inhibitor, PD098059 (PD, (3)) which leads to the inhibition of ERK activity. The growth of OITs, which occurs in insulin-only basal medium, was also inhibited by PD. These results suggested that basal ERK was permissive for proliferation. PT which inhibits the growth-stimulatory effect of cAMP, also inhibited ERK activity indicating that a $G\alpha i$ protein-coupled pathway was involved in ERK regulation and was permissive for cAMP mitogenesis. Examination of the c-jun kinase (JNK) pathway indicated that like ERKs, JNK activity was not affected by cAMP in normal or PDT cells but was inhibited by cAMP in OIT. Our studies showed that neither cAMP inhibition (OIT) nor stimulation (normal, PDT) of proliferation was reflected in a corresponding effect on the most likely kinase target pathway, the ERK pathway. Inhibition of JNK activity in OIT suggested that other MAPKs belonging to the stress activated protein kinase family might be affected by cAMP. Interestingly, in T lymphocytes cAMP was inhibitory to JNK but not ERK (4) and cAMP inhibition of JNK correlated with thrombin-induced inhibition of vascular smooth muscle cell growth (5).

The inhibitory effect of PT on cAMP-stimulated growth and ERK suggested that a $G\alpha i\beta\gamma$ protein-mediated pathway could be involved but the only potential G protein-coupled receptor ligand present in the medium was insulin. Insulin was present at a superphysiological concentration (10 μg/ml), however, and was probably binding to the insulin-like growth factor receptor I (IGF-I) (6) which can reportedly signal through G protein-coupled pathways (7). Thus, PT could

potentially inhibit IGF-I receptor pathways stimulated by superphysiological insulin that maintains basal ERK activity which is permissive for cAMP mitogenesis. This explanation raises the possibility that the inhibitory effect of PT on cAMP-stimulated proliferation occurs indirectly i e. not on a pathway directly activated by cAMP.

Other recent studies have shown that cAMP can activate ERKs through rap1-mediated activation of B-raf in contrast to its negative effect on ERK activation via inhibition of raf-1 (8, 9). The relative abundance of B-raf or raf-1 could then act as a switch that determines tissue specific regulation of the ERK pathway by cAMP. This raises the possibility that the differential expression of these factors could cause a switch in the cAMP response as we have observed between normal mammary epithelium and hormone-independent mammary tumors (OIT).

Body

Materials and Methods

Reagents. Cell culture: Ham's F-12, Medium 199, and Dulbecco's Modified Eagle's medium (DMEM) were from GIBCO/BRL (Grand Island, NY); collagenase (CLS Type 2) was from Worthington Biochemical Co. (Freehold, NJ), Percoll was from Pharmacia Biotech (Piscataway, NJ). Rat tail collagen, solubilized in acetic acid, was prepared as described previously (10). *Antibodies:* ERK 1 (C-16), ERK 2 (C14), EGF receptor, IGF-1 receptor, raf-1, B-raf, jun kinase (JNK) and phospho-specific JNK were from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-specific p44/42 (pERK 1 and pERK 2, was from Promega (Madison, WI); CREB, phospho-specific CREB, phospho-specific p38 and p38 was from New England Biolabs, phospho-tyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). *Biochemicals:* MEK1 inhibitor, PD 098059, and EGFR receptor phosphorylation inhibitor, Compound 56, were from Calbiochem (San Diego, CA). Protein A and G agarose, dibutryl cyclic AMP (cAMP), pertussis toxin, 1-oleoyl lysophosphatidic acid (LPA) were from Sigma Chemical Co. (St. Louis, MO)). LPA was prepared by brief sonication of an aqueous suspension in Saline A containing bovine serum albumin (1 mg/ml). Gst-c-jun fusion protein substrate was prepared using a fusion construct provided by Roger Davis. EGF was from Collaborative Research (Waltham, MA).

Cell culture and tissues. Pregnancy-dependent mammary tumors were propagated *in vivo* by intrafat pad transplants in estrogen and progesterone treated (injection or pellet) DDD mice. Ovarian-independent mammary tumors were raised by subcutaneous transplantation of tumor pieces in virgin DDD mice. These hormone-independent tumors grow rapidly in virgin hosts. Normal tissues were from mature virgin Balb/cAnNCrlBR mice obtained from Charles River.

Normal and tumor tissues were dissociated with collagenase (0.1%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (10). For growth experiments, cell organoids were cultured for 10 days within collagen gels as described (10). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham's F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 μg/ml insulin, 100 U/ml soybean trypsin inhibitor, 1 μg/ml a-tocopherol succinate and

other additives as indicated. Cell number was determined by fluorometric DNA assay using diaminobenzoic acid (11) and standard curves using diploid tumor cells.

Cell cultures for kinase assays were established as above using 10 or 6 cm culture dishes with 5 to 10 million cells per culture. For some experiments, cells were cultured on the surface of collagen I-coated plates. Normal cells were cultured for 4-6 days in serum-free medium (above) prior to the addition of test factors. Some OIT were first precultured for 24-48 hrs in 2% porcine serum before washing and switching to serum-free medium for the remainder of the culture period. cAMP (100 μg/ml) was added in combination with the cAMP phosphodiesterase inhibitor RO-20-1724 (10^{-5} M, Calbiochem). Pertussis toxin (100 ng/ml) was added overnight (to allow activation by the cells) prior to the addition of test factors.

Preparation of Cell Extracts. After incubation for various times, cultures were terminated by aspiration of the culture medium followed by blotting of the gels on filter paper and transfer of the dehydrated gels to 0.6 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerophosphate, 40 mM PNPP, 200 mM sodium orthovanadate, 100 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin. The lysates were mixed by vortexing and left on ice for 60 min. before centrifugation (10 min, 13,000 x g, 4° C). Nuclear extracts were prepared with the above buffer containing 0.4 M KCL. Cells cultured on collagen-coated plates were terminated by direct addition of lysis buffer to the plate on ice. Supernatants were used for immunoprecipitations and western blot analysis. Protein concentration was determined using the BCA assay by Bradford.

Western Blotting. Sample lysates containing 20 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBS buffer containing 5% (w/v) dry milk (or 1% BSA for phosphotyrosine antiserum) and 0.1% Tween and incubated with the manufacturer's recommended dilution of antibody (dilutions were adjusted as necessary). For all immunoblotting, a horseradish peroxidase-conjugated secondary antibody was utilized to allow detection of the appropriate bands using enhanced chemiluminescence (ECL, Amersham Corp.). Membranes were stripped for reprobing with another antisera by incubating at 50° C for 30 min. in Tris (63 mM, pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol then washed in PBS and reblocked. For data analysis, ECL-detected bands were scanned using a Molecular Dynamics Personal Densitometer using ImageQuant software.

Results and Discussion

Objectives:
The objectives of the present studies were to 1) examine the role of insulin/IGF-I in PT-sensitive cAMP stimulation of proliferation, 2) determine if B-raf is expressed and if the B-raf/raf-1 ratio differs between normal MEC and OIT, 3) determine if another MAPK, p38, was activated differentially in normal and tumor mammary epithelium by cAMP and other mitogens including, 4) examine the effect of a Gαiβγ-coupled lipid mitogen lysophosphatidic on kinase pathways

1. Insulin requirement for cAMP mitogenesis. (Task 2).

Effect of cAMP and PT on growth in the presence and absence of insulin:
PT inhibited cAMP stimulation of proliferation in serum-free medium containing only superphysiological insulin (10 ug/ml). Insulin at this concentration is a permissive requirement for optimal growth of mouse mammary epithelium in response to all known growth-stimulating factors (12, 13). We asked if the inhibitory effect of PT was due to the inhibition of insulin-like growth factor I receptor (IGF-IR) mediated signaling since insulin at the concentration used can bind to the IGF-IR (6). The effect cAMP (100 μg/ml) and PT (10 ng/ml) on growth and kinase activation was compared in normal MEC cultured in collagen gels for 12 days in serum-free medium in the absence (I0) or presence (I10) of insulin (10 μg/ml) (**Fig. 1**). In I0 medium, cAMP treatment was only weakly stimulatory to growth relative to basal medium controls where cell number diminished. Compared to the starting cell number (OT) there was no net increase in cell number showing that cAMP stimulated only enough proliferation to compensate for cell loss occurring in the absence of insulin. The addition of PT, however, lowered the cell number showing that PT has an effect even in the absence of insulin. In contrast, cAMP stimulated multifold growth in I10 medium that was strongly inhibited by PT. We show that cAMP requires superphysiological insulin (lowering the insulin concentration diminishes the proliferative effect, data not shown) to stimulate proliferation but PT sensitivity is insulin-independent. That PT can inhibit the proliferative effect of cAMP in the presence and absence of superphysiological insulin implies that the PT-sensitive step is not related to insulin receptor or IGF-IR activation.

Effect of cAMP and PT on kinase activity in the presence and absence of insulin:
When ERK activity was examined by western immunoblotting of cell extracts with phopho-specific ERK antiserum, basal activity was similar (slightly higher in I10) and inhibited by PT in both I0 and I10 media (**Fig 2**). These results imply that an insulin/IGF-I pathway does not directly regulate ERK and is not involved in the PT response, in agreement with the proliferation assays (Fig. 1). cAMP inhibited ERK activity as previously observed in I10 as well as I0 medium (**Fig 2**). PT inhibited <u>basal</u> ERK activity in both media but PT inhibition in the <u>presence of cAMP</u> was almost complete in I10 medium whereas in I0 medium it had a slight effect. Total ERKs were the same level in all lanes as revealed by immunoblotting with ERK antisera. Thus, PT inhibited ERK activity to a greater extent in I10 medium containing cAMP suggesting that cAMP sensitizes the ERK pathway to PT via an interaction with an insulin/IGF-I pathway. This level of inhibition of the ERK pathway may inhibit proliferation since we know that cAMP mitogenesis is inhibited by inhibiting ERK activation with the MEK inhibitor PD98059. The I0 medium data indicate, however, that PT inhibition of ERK activity and proliferation is not dependent upon the co-activation of an insulin/IGF-I pathway. But, in the presence of cAMP, a PT sensitive insulin/IGF-I pathway may be involved that can lead to almost total inhibition of growth and ERK activation. One caveat for these experiments is that the cells used for kinase assay were precultured in 2% serum for the first 48 hrs to promote growth prior to switching to serum-free medium for another 4 days. The basal ERK activity in I0 might be a continued response to this serum exposure so we plan to repeat these experiments under completely serum-free conditions.

The effect of cAMP on the activation of the stress associated protein kinases, p38 and JNK was also examined I10 serum-free medium to compare the response of these kinases to the ERKs. Cells were cultured and treated with cAMP and PT as described above and kinase activation was assessed by western immunoblotting of cell lysates with phospho-specific antisera against JNK and p38 (**Fig 3**). Unlike ERKs, p38 activity was unaffected by cAMP or PT while JNK activity was slightly inhibited by PT or cAMP (30% maximal inhibition in the presence of cAMP and PT) although examination of the variation in this inhibitory effect in other experiments indicates that this result is probably not significant. Therefore, the effect of PT on growth correlates best with an inhibitory effect on the ERK, not the JNK or p38 pathways.

IGFI receptor expression and activation:
Since cAMP or PT may affect the activation of the IGF-IR, studies have been initiated to monitor the activation of IGF-IR in cultured normal and OIT cells by immunoprecipitation of the receptor followed by western immunoblotting with antiphosphotyrosine antisera. Using IGF-IR antisera from Santa Cruz we can detect IGF-IR by direct western blotting of of cell extracts and no consistent difference in the level of receptor can be discerned between normal MEC and OIT in four different experiments. We have not been able to detect any appreciable tyrosine phosphorylation of the IGF-IR in cells cultured in basal insulin-containing medium in the presence or absence of cAMP. We plan to try alternative commercially available antisera and if no effect is observed, examine the phosphorylation state of the insulin receptor and the effect of IGF-I substitution of insulin.

2. CREB activation (new work)

We have begun to look at downstream factors that may be activated by multiple kinase pathways and serve as endpoints to measure the relative contributions of different pathways to transcriptional and biological responses. The effect of cAMP and PT on the phosphorylation of the transcription factor CREB which is a substrate for cAMP/PKA and RSK is under examination but western immunoblotting of nuclear extracts with phosphospecific CREB antisera does not reveal any significant effect of cAMP on CREB phosphorylation, the level of which is barely detectable in our hands (not shown). CREB antisera do show the presence of the protein in the normal MEC used for these experiments. This data is very preliminary and more work is needed in optimizing the procedure for obtaining nuclear extracts and immunoprecipitating CREB. We do observe activated ATF-1 in basal medium cultures with no modulation by cAMP which suggests that this family of transcription factors, more particularly ATF-2, should be examined in parallel with CREB. We plan to continue to optimize conditions to assay transcription factor activation in normal cells and extend this work to mammary tumors.

3. Raf expression in normal and tumor cells (new work)

We also began studies examining the presence of B-raf and raf-1 in normal MEC and OIT to determine if the ratios of these kinases, which lie upstream of MEK in the ERK pathway, differed in cells with different proliferative responses to cAMP. We might predict that if cAMP inhibits proliferation that the level of raf-1, which is inhibited by cAMP-activated PKA, will be higher than the level of B-raf, associated with PKA stimulation of proliferation. The opposite may be

occur in the OIT. When the relative levels of raf-1 and B-raf were compared in normal and tumor cells cultured in basal serum-free medium (always with I10 unless otherwise indicated), the opposite effect was observed (**Fig. 4**). It was interesting to find that both kinases are expressed in normal and tumor cells, that but relatively more B-raf relative to raf-1 was found in the OIT compared to normal or PDT cells. In other experiments the elevated level of B-raf is not as dramatic as shown in Fig. 4 but it is safe to conclude that B-raf underexpression relative to raf-1 is not associated with the inhibitory effect of cAMP on proliferation.

4. Comparison of p38 activation in normal and tumor mammary epithelium. (Task 1)

Lysates from the same collagen gel cultures as above (no. 3) were screened with phospho-specific p38 antisera to compare the level of activated p38 in these cells. We find that the level of active p38 in cells cultured within collagen gels, in basal serum-free medium is elevated in OIT compared to normal MEC and PDT (**Fig. 5**). Total p38 assessed by immunoblotting with p38 antisera showed that the level of enzyme was similar in normal and tumor cells. Thus, an elevation in p38 activity may be significant for the OIT phenotype and suggests that the regulation of this enzyme is altered in late stage breast tumors. The effect of cAMP on p38 activation was examined in normal cells cultured within collagen gels or on the collagen-coated dishes. In 3 experiments each, no effect of cAMP treatment (30 min - 60 min) on p38 phosphorylation was observed as assessed by western immunoblotting with phospho-specific antiserum (not shown). Examination of the effect of cAMP on p38 phosphorylation in 4 cultured OITs (3 within collagen gels, 1 on collagen-coated dishes) also showed that cAMP did not affect the phosphorylation of p38 (not shown). Thus, cAMP does not significantly activate or inhibit this pathway in normal MEC or OIT. Three PDT tumors cultured within collagen gels have been examined and indicate that cAMP elevates p38 activity about 1.5-2-fold but these results need to be confirmed. These results indicate that basal p38 activity may be altered in OIT but that the differential effect of cAMP on proliferation of normal MEC and OIT does not correlate with an effect on p38 activity.

5. Effect of lysophosphatidic acid on kinase activity in normal MEC and OIT. (Task 1)

Lysophosphatidic acid (LPA) is a phospholipid mitogen that selectively affects normal and tumor MEC growth in the same manner as cAMP (2). Its mitogenic effect is similarly PT sensitive. All the evidence we have obtained thus far indicates that cAMP mitogenesis is not directly associated with the activation of ERK, JNK, or p38 activity in normal or tumor MEC with the possible exception of p38 activation in PDT. However, the proliferative effect of cAMP is dependent upon basal ERK activity as shown by the inhibitory effect of PT and the MEK inhibitor PD98059. We have attempted to gain further insight into growth- stimulatory and growth-inhibitory pathways by comparing the effect cAMP with that of LPA on kinase pathways.

The effect of inhibitors of LPA on activation of the ERK, JNK, and p38 pathways was investigated in normal MEC and OIT. Normal MEC and OIT cells were cultured on collagen-coated dishes, in serum-free medium for 8 days and LPA was added in time course and dose/response studies. LPA stimulated the activity of all of these kinases at an optimum concentration of 0.05 mM, which coincides with the

optimum effect on proliferation, with activity peaking at a duration of exposure of 4-10 min. **Fig. 6. (A, B, C)** is a summary of 3-6 experiments in which activated kinase was detected by immunoblotting with phospho-specific antisera to these kinases or in the case of JNK, also confirmed by direct assay of kinase activity in immunoprecipitates. We have shown that detection of activated ERK by immunoblotting with phospho-specific antisera correlates with direct assay of kinase activity in ERK immunoprecipitates (Appendix 2, (14)). Bound antisera was detected by ECL and bands quantitated by scanning densitometry. Data are normalized to basal (i.e. fold above basal, and represent arbitrary units). LPA treatment activated ERK and p38 relatively more than JNK. LPA activation of ERK was inhibited by PT (**Fig. 6D**) indicating a Gαi protein-coupled pathway. In contrast, PT did not inhibit LPA-induced activation of JNK or p38. So LPA can activate multiple kinases by PT-dependent and -independent pathways.

LPA stimulation of signaling in a variety of cell lines has been shown to occur through ligand-independent activation of the EGF receptor (15-18). Transactivation is shown by LPA-induced phosphorylation of the receptor, and the demonstrated inhibition of LPA activation of ERKs by specific inhibitors of EGF receptor phosphorylation (and activation) such as Compound 56 (Calbiochem). As shown in **Fig. 6**, we examined the effect of Compound 56 (C56) on LPA activation of kinases and observed a partial inhibition of ERK. However, there was no effect on JNK or p38 activity. Shown for comparison is the effect of EGF and the inhibitory effect C56 on EGF activation of ERK and JNK but a lessor inhibition of p38. The inhibitory effect of C56 on ERK activity was inconsistent (2/4 experiments) as was an observed an elevation in EGF receptor tyrosine phosphorylation in LPA-stimulated cultures (not shown). In comparison to EGF activation of EGF receptor tyrosine phosphorylation, LPA's transactivation of the EGF receptor is about 10-fold lower. In the same experiments, consistent C56 inhibition of EGF-induced EGF receptor phosphorylation was observed. Thus, the kinase experiments suggests that LPA couples to multiple kinase pathways but only weakly through transactivation of the EGF receptor. Only the ERK pathway is PT-sensitive which is reminiscent of the effect of PT on these kinases in cAMP-treated cells. But unlike cAMP, LPA activates the ERK pathway.

The effect of PT, PD98059 and the EGF receptor phosphorylation inhibitor, Compound 56 (C56), on LPA-induced proliferation of normal MEC was also assessed (**Fig. 7**). PT and PD98059 inhibited the effect of LPA on proliferation consistent with their inhibitory effect on LPA-induced ERK activity indicating that activation of the ERK pathway is important for LPA mitogenesis. We also observed a partial inhibitory effect of C56 which is consistent with its partial inhibitory effect on ERK activity. Dose-response studies were performed with C56 and the effect is dose-dependent and maximum at the indicated concentration of 100 μM (**Fig. 7**). At this time we suspect that this drug may act via an EGF receptor-independent mechanism as well or our ability to detect ligand-independent EGF receptor phosphorylation is not sensitive enough. Clearly, studies examining the effect of a dominant negative EGFR construct on LPA-induced responses would be desirable. However, results obtained with OITs (below) suggest that LPA can activate ERKs independently of EGF receptor transactivation. Toxicity is apparently not an issue since the cultures do not show signs of cell degeneration.

We have begun studies examining the effect of LPA on OIT and the data are preliminary. However, some conclusions can be drawn. To recapitulate, OIT growth is autonomous and independent of EGF in our culture system. EGF independence is explained by the low to undetectable level of EGF receptors in these tumors as assessed by western immunoblotting with EGF receptor antiserum under assay conditions where the receptor is easily detected in normal MEC (not shown). We have tested C56 on OIT in which growth is inhibited by LPA and find that, as expected, it does not reverse the inhibitory effect of LPA on growth (**Fig. 8**), the stimulatory effect of LPA on ERK activity (**Fig. 9**) or JNK activity (**Fig. 10**). Thus, LPA, at least in OIT, can activate MAP kinases and inhibit proliferation independently of EGF receptor transactivation. What is interesting is the comparison of the effect of cAMP and LPA on the activity of MAP kinases. Although both factors are inhibitory to growth, cAMP has no effect (ERK, p38) or is inhibitory (JNK) to MAP kinases while LPA is stimulatory (ERK, JNK). The effect of LPA on p38 activity in OIT is under investigation. We are drawn to the conclusion that the inhibitory effect of LPA and cAMP on proliferation is independent of direct stimulatory or inhibitory effects on MAP kinases (ERK, JNK) associated with proliferation control by mitogenic growth factors and cAMP in other systems.

Research Accomplishments

1. Begun examination of the role of a PT-sensitive insulin receptor or IGF-I receptor activation in cAMP-induced mitogenesis.

2. Compared the level of raf-1 and B-raf expression in normal and tumor mammary epithelium.

3. Examined the level and regulation of p38 MAP kinase in normal and tumor mammary epithelium.

4. Compared the effect of cAMP and lysophosphatidic acid on MAP kinase activation in normal and tumor mammary epithelium.

Reportable Outcomes

Manuscript (Appendix 2): Xing, C and Imagawa, W (1999) Altered MAP Kinase (ERK-1,-2) Regulation in Primary Cultures of Mammary Tumor Cells: Elevated Basal Activity and Sustained Response to EGF. Carcinogenesis 20, 1201-1208

Conclusions

1. Preliminary results indicate that PT sensitive inhibition of ERK activity is independent of superphysiological insulin but is enhanced by cAMP only in the presence of superphysiological insulin. The inhibition of basal ERK activity by PT in insulin-containing medium bolsters our previous conclusion that basal ERK activity is permissive for cAMP mitogenesis and suggests that the PT-sensitive component of cAMP mitogenesis may be due to co-stimulation by $G\alpha i$ protein-coupled insulin or IGF-I receptor activation. In the next year, the effect of cAMP and PT on IGF-I and insulin receptor activation will be explored.

2. Regulation of CREB transcription factor activation has been initiated but

although no firm results are in hand we plan to accelerate the examination of transcription factor activation (ATF-2, ELK-1, c-jun) to determine if cAMP pathways converge at this level of regulation. This was not included in the previous statement of work but our findings indicate that we must examine factors downstream of kinase activation in order to gain insight into cAMP's differential effect on proliferation between normal and tumor cells.

3. The examination of raf expression patterns revealed that shifting ratios of raf-1 to B raf do not explain the different mitogenic effects of cAMP on normal MEC and mammary tumors. This suggest that the mechanisms responsible for the differential effect of cAMP on normal and tumor mammary epithelium lies either downstream of raf in signaling pathways or is not raf-dependnet except in a permissive manner.

4. We have examined the regulation of p38 MAP kinase and find no evidence for cAMP or PT regulation in normal MEC or OITs although basal activity appears to be elevated in OITs. Therefore, the effect of PT on growth correlates best with an inhibitory effect on the ERK, not the JNK or p38 pathways in normal MEC. The significance of elevated p38 activity in OITs is not known but proliferation experiments using the p38 pathway inhibitor SB202190 are planned to assess the requirement of this pathway for proliferation control by cAMP.

5. The effect of LPA on signaling has been compared to cAMP. In spite of the similar effects these two factors have on the proliferation of normal and tumor MEC, we find that they have markedly different effects on kinase activation. LPA stimulates the activity of ERK, JNK, p38 while cAMP is inhibitory to ERK in normal MEC and JNK in OIT. The effect of LPA on kinase activity is PT-dependent for ERK but not JNK or p38. These results strengthen the interpretation that the positive and negative effect of cAMP on proliferation are not related by corresponding parallel effects on MAP kinases. However, a threshold level of ERK appears to be permissively required for cAMP mitogenesis. The pertussis toxin sensitivity of cAMP mitogenesis may be due to activation of the insulin or IGF-I receptor but other PT-sensitive pathways cannot be excluded. Overall these findings direct our attention to protein kinase A pathways and substrates including transcription factors. The monitoring of transcription factor activation by cAMP, EGF (a known strong activator or ERKs), LPA, and PT is planned to look for crosstalk at this level.

6. These findings contribute to our knowledge of cAMP regulation of growth in normal and tumor MEC. The identification of cAMP-mediated pathways and genes that are responsible for the inhibition of mammary tumor growth can lead to the production of chemicals or probes of prognostic or therapeutic use.

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Appendices

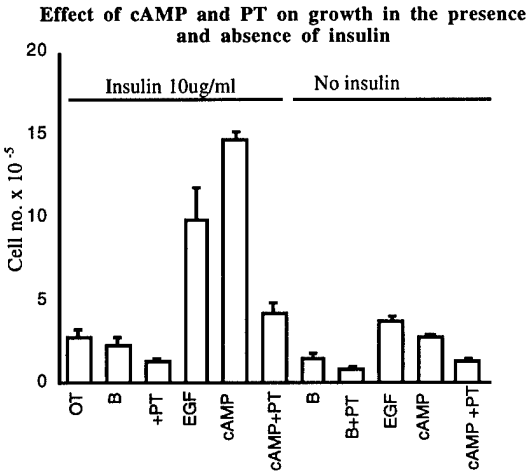

Figure 1. Effect of insulin on cAMP mitogenesis. Normal MEC were cultured for 10 days in serum-free medium with or without insulin (10 μg/ml). cAMP (100 μg/ml), PT (10 ng/ml), or EGF (10 ng/ml) were added as indicated. Mean and SD or triplicates is plotted.

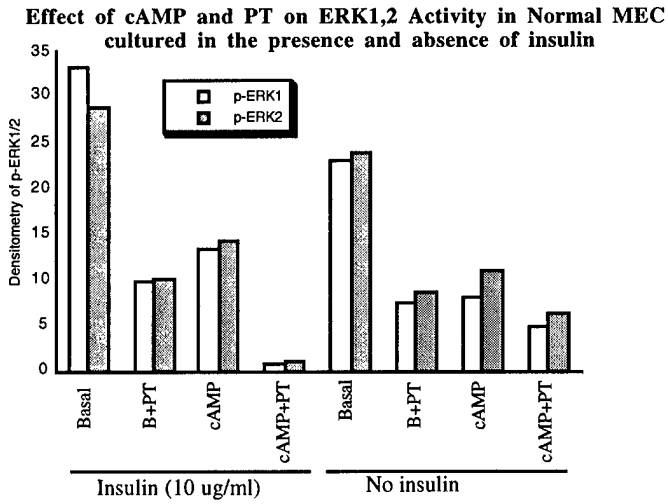

Figure 2. Effect of insulin on cAMP and PT inhibition of ERK activity in cultured normal MEC. Cells were cultured in collagen gels for 10 days (2% serum was added for the first 24 hrs prior to switching to serum-free medium with or without. insulin (10 μg/ml). cAMP treatment was 30 min., and PT was added overnight. Activated ERK was determined by western immunoblotting with antiphospho-ERK antisera. Relative intensity of resultant bands detected by ECL was determined by scanning densitometry.

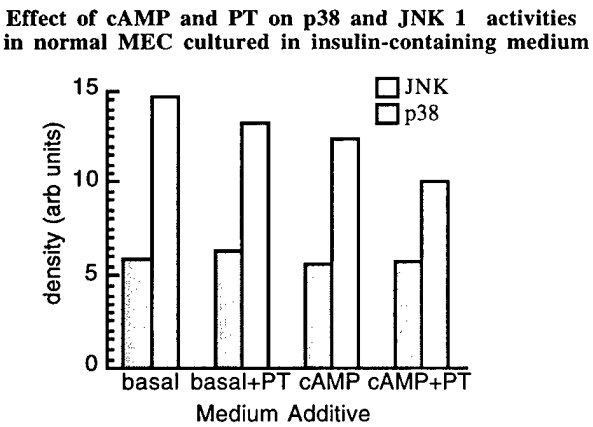

Figure 3. Effect of cAMP and PT on p38 activity. Normal MEC were cultured as in Fig 2. Lysates were western immunoblotted with phospho-specific antiser to JNK and p38. Relative intensity of bands detected by ECL was determined by scanning densitometry.

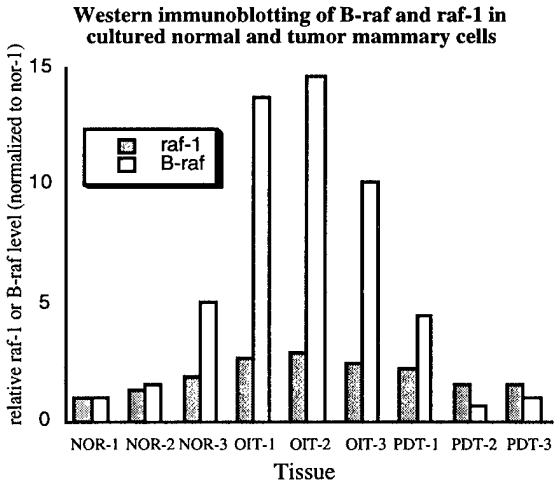

Figure 4. Relative level of B-raf and rat-1 in normal MEC and mammary tumors (OIT, PDT). Cells were cultued within collagen gels in serum-free basal medium containing insulin (10 μg/ml) for 10 days. At this time, cells lysates were prepared and raf-1 and B-raf detected by western immunoblotting. All samples were analyzed togethere on the same blot which was probed by raf-1 antisera after stripping of B-raf. Densitometry was performed as described and band density normalized to Nor-1.

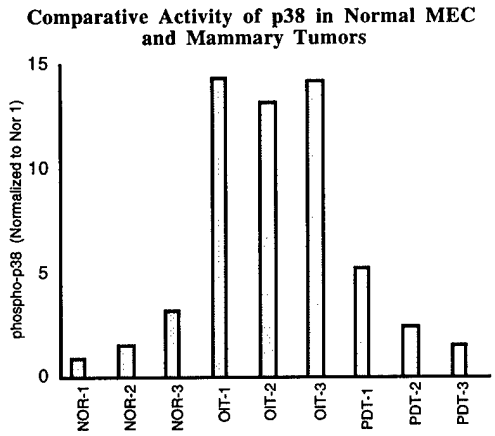

Figure 5. Relative level of activated p38 in normal MEC and mammary tumors (OIT, PDT), Cells were cultured as described in Fig 4. Cell lysates were subjected to western immunoblotting with phospho-specific antiserum to p38. After ECL detection of bands, relative band density was determined by scanning densitometry and normalized to Nor-1.

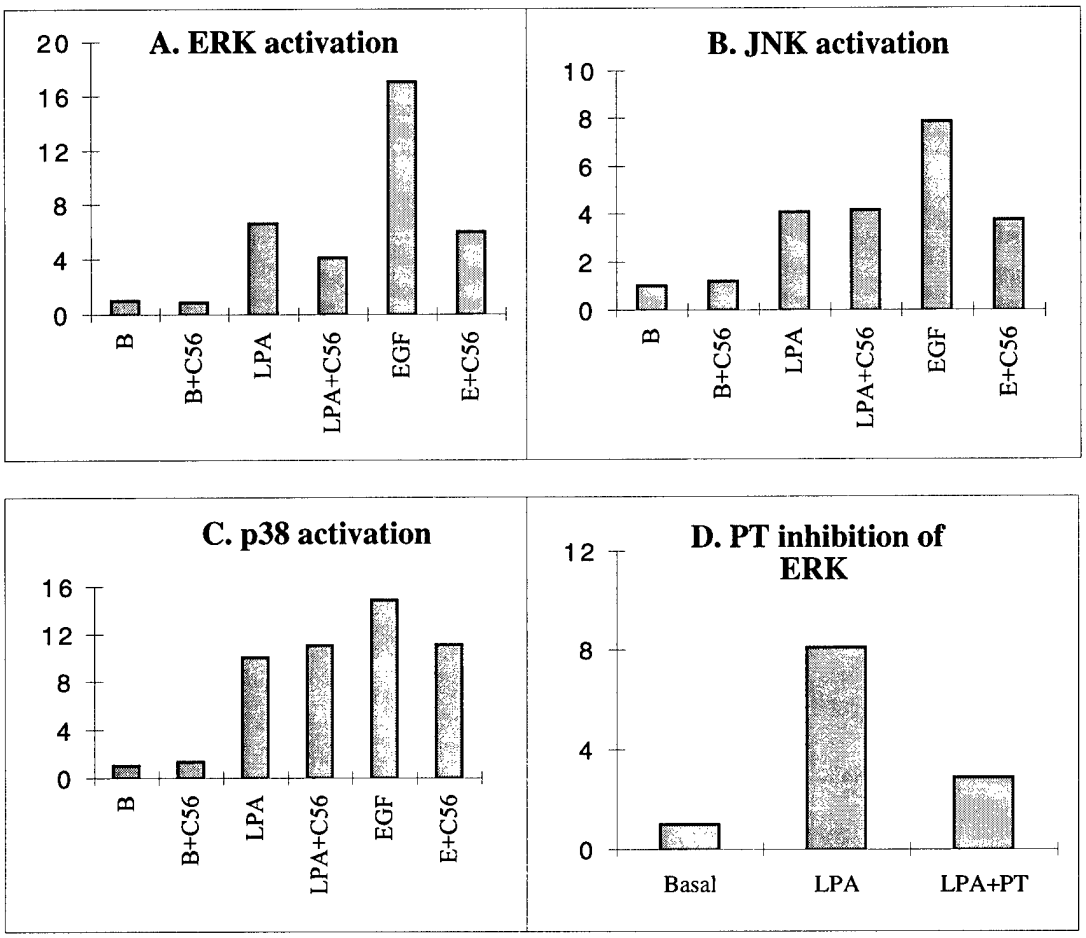

Figure 6 (A,B,C,D)
Effect of LPA on ERK, JNK, and p38 activation in normal MEC. Ave of 4-5 experiments is plotted as fold of basal. Values are derived from densitometry of bands detected after western immunoblotting witl phospho-specific antisera or and/orJNK kinase assay. LPA (0.05 mM) treatment was 10 min., EGF 4 min. C56 (100 mM) was added 2 hrs prior to LPA and PT (100 ng/ml overnight.

Effect of inhibitors on LPA mitogenesis

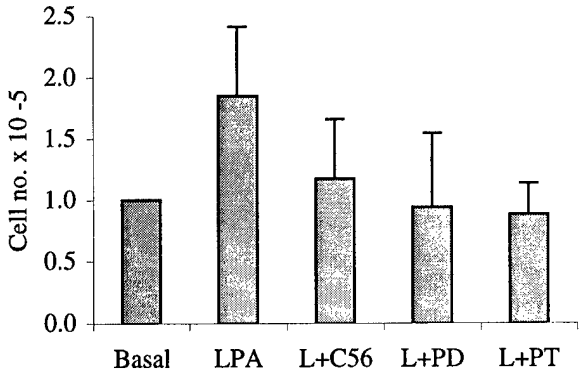

Figure 7. Effect of Inhibitors on LPA mitogenesis. Normal MEC were cultured within collagen gels for 10 days with the above additives: LPA (0.05 mM), Compound 56 (C56,100 uM), PT (10 ng/ml), PD98059 (50 μM). Average and SD of 3 experiments, nomalized to basal.

Effect of Compound 56 on LPA mitogenesis in OIT

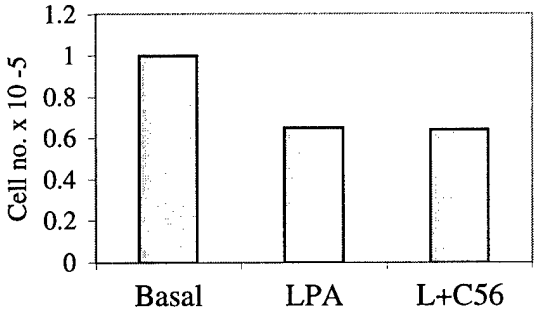

Figure 8. Effect of Compound 56 on LPA inhibition of growth in OIT.
Plotted is mean cell numbernormalized to basal from a single experiment done in triplicate.
Cells were cultured for 10 days in collagen gels. LPA (0.05 mM), C56 (100 μM).
C56 does not affect the inhibitory effect of LPA on growth

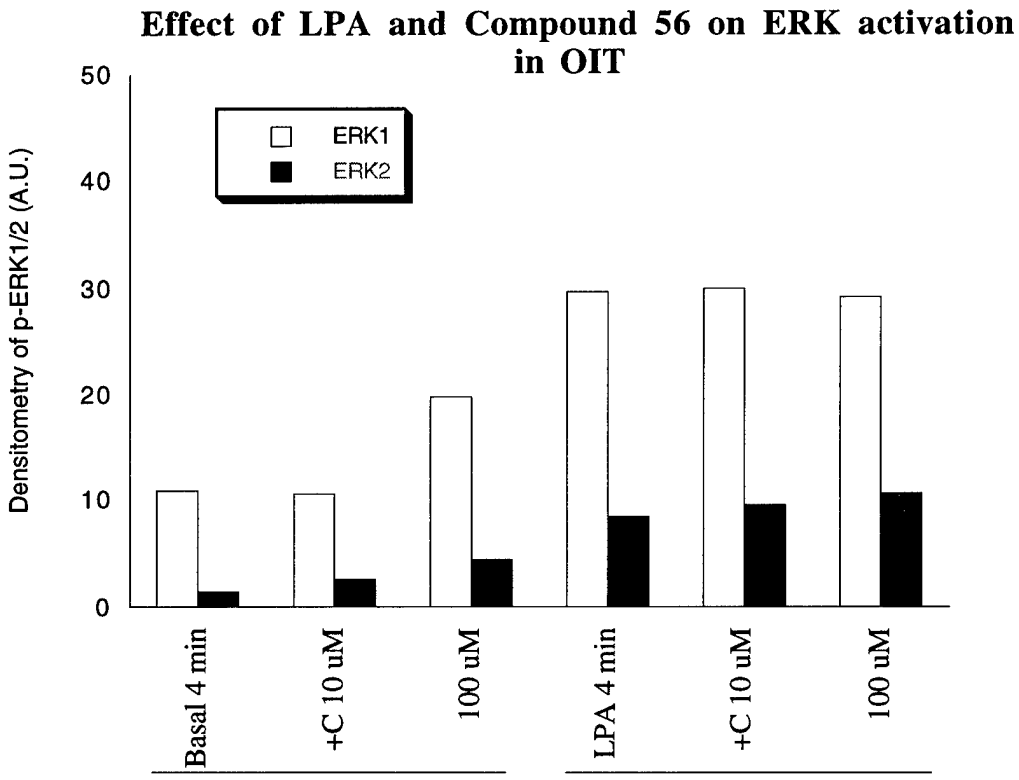

Figure 9. Effect of LPA and Compound 56 on ERK activation in OIT. Results of one experiment is plotted. Cultured OIT were treated with LPA (0.05 mM) for 4 min., C56 (10 and 100 μM) was added one hour before LPA . Cells were lysed and ERK activation examined by western immunoblotting with phospho-specific ERK antisera.

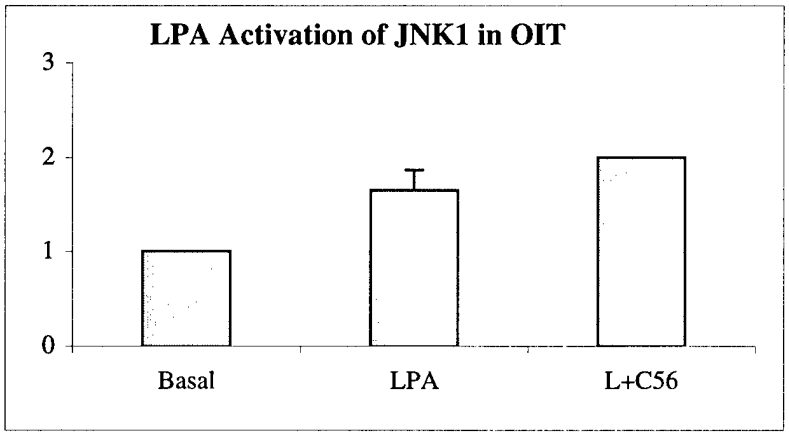

Figure 10. Effect of LPA on JNK 1 activity in OIT. Average of two experiments is plotted, normalized to basal. Doses treatment schedule are the same as in Fig 9. C56 was added at 100 μM.

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Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF

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An elevation in total MAP kinase activity and expression has been observed in breast cancer tissue. However, the mechanisms underlying these changes in kinase activity and regulation by growth factors are not well characterized. In these studies, the effect of the potent mammary mitogen, epidermal growth factor (EGF), on the activation of the mitogen-activated protein kinases, ERK1 and ERK2 (extracellular regulated protein kinases 1 and 2, respectively), was compared in primary cultures of normal mouse mammary epithelial cells and in a hormone-responsive mouse mammary tumor. In normal epithelium, EGF stimulated an early rise in ERK activity at 4 min followed by a rapid decline, whereas a sustained (1 h) elevation of ERK activity was observed in the tumor cells. The time course of ERK activity in both cell types coincided with the phosphorylation state of the EGF receptor, suggesting that altered regulation of EGF receptor phosphorylation or EGF receptor turnover produces an enhanced ERK response to EGF in tumor cells. The MEK inhibitor, PD 098059 inhibited EGF-stimulated proliferation and ERK activity in a parallel, dose-dependent manner showing that ERK activation is at least permissive for the proliferative response to EGF. In addition, tumor cells showed a 4-fold elevation in basal (or ligand-independent) activity over normal cells without an increase in total enzyme level, and a preferential activation of ERK1 by EGF. These EGF-dependent and -independent changes in ERK regulation in the hormone-responsive mammary tumor underscore how multiple alterations in the regulation of this pathway may play a role in mammary tumorigenesis.

Introduction

Epidermal growth factor (EGF) is a potent mitogen for normal and tumor mammary epithelial cells. Overexpression of EGF and its receptor (EGFR or ErbB-1) has been observed in breast cancer, raising the possibility of potential autocrine growth regulation as observed in some breast cancer cell lines (1). Elevations in EGFR levels can also play a role in mammary tumor progression from hormone dependence to hormone independence, as shown in mouse mammary tumors (2) and in human breast cancer cells when the EGFR is overexpressed (3). In addition, an elevated level of EGFR is associated with a poor prognosis in breast cancer (1). The EGF receptor is a transmembrane tyrosine kinase that is activated by tyrosine autophosphorylation after ligand-induced dimerization (4). The ligand-bound receptor is capable of phosphorylating multiple signal transduction molecules, leading to the activation of multiple signal transduction pathways including protein kinase cascades (5). One of these kinase cascades is the *ras–raf* pathway leading to the activation of the extracellular regulated protein kinases (or ERKs), one of the mitogen-activated protein (MAP) kinase cascades. This pathway has been the subject of intense interest because of its role in the regulation of proliferation, differentiation and cell–matrix interactions. ERK1 and ERK2 are dually phosphorylated on threonine and tyrosine by the upstream MAP kinase kinase, MEK. ERKs then phosphorylate and activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases leading to positive or negative regulation of signaling cascades (5).

Disruption of the regulation of the ERK pathway can predispose cells to undergo tumorigenic conversion as illustrated by the *ras* oncogene (6) which lies upstream of ERK, and transfection studies showing that constitutively active mutants of MEK can lead to *in vitro* transformation, increased sensitivity to, or independence from growth factors and tumor formation *in vivo* (7,8). Recent studies have shown an increase in the level and kinase activity of ERKs in human renal cell carcinoma (9) and breast cancer (10), indicating that deregulation resulting in overstimulation of this pathway may play a role in tumorigenesis.

These latter studies in primary human tumors did not address the regulation of the activation of the ERK cascade by exogenous factors. It was not possible to discern if an apparent constitutive elevation in basal activity was due to an inherent alteration in the regulation of the pathway or if the pathway was more sensitive to stimulation by an exogenous ligand. In our studies, we have sought to directly compare the effect of EGF on the activation of the ERKs in primary cell cultures of normal and tumor mammary epithelium to better recognize alterations that may occur during tumorigenesis. Primary culture using a biomatrix-based culture system was chosen to more closely approximate the *in vivo* state and as an alternative to immortalized cell lines adapted for growth on a plastic substrate. These experiments show that the mitogenic effect of EGF in normal and tumor mammary epithelium is dependent, at least in part, on ERK activation. Furthermore, mammary tumor epithelium may exhibit an elevation in basal ERK activity and sustained ERK activation by EGF, the latter sustained activation reflecting a difference in the regulation of EGF receptor activity. This altered regulation of the MAP kinase pathway may be an example of an alteration in regulation by growth factors that may occur in some breast cancers impacting both tumor growth and progression.

Materials and methods

Reagents

Cell culture: Ham's F-12, Medium 199 and Dulbecco's Modified Eagle's medium (DMEM) were from Gibco BRL (Grand Island, NY); collagenase

Abbreviations: EGF, epidermal growth factor; ERK, extracellular regulated protein kinase; MAP kinase, mitogen-activated protein kinase.

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(CLS Type 2) was from Worthington Biochemical (Freehold, NJ), Percoll was from Pharmacia Biotech (Piscataway, NJ); rat tail collagen, solubilized in acetic acid, was prepared as described previously (11). Antibodies: ERK1 (C-16), ERK2 (C-14), and EGF receptor (EGFR) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-specific p44/42 (ERK1 and ERK2) antibody was from Promega (Madison, WI); phospho-tyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Biochemicals: MEK1 inhibitor PD 098059 was from Calbiochem (San Diego, CA); MAP kinase substrate peptide containing the MAP kinase consensus phosphorylation sequence (amino acids 95–98 of MBP) was from Santa Cruz Biotechnology; [γ-^{32}P]ATP was from DuPont-NEN. EGF was from Collaborative Research (Waltham, MA) and Protein A agarose was from Sigma (St Louis, MO).

Cell culture and tissues

Mammary tumors were raised from intrafat pad transplants of an undifferentiated mammary carcinoma derived after transfection of primary mammary epithelial cells from virgin Balb/c mice with the BAG (12) retroviral vector containing a mouse Gαi2 cDNA insert. This tumor arose originally from transfected cells transplanted into cleared fat pads and is stored as a frozen stock. Tumors are raised in virgin hosts *in vivo* by transplantation into the fat pad. In this tumor, the retroviral insert appears to have undergone rearrangement and a full length retroviral transcript is not detectable by northern analysis (unpublished observations). *In vivo*, this tumor can grow in ovariectomized hosts, while *in vitro*, progesterone and prolactin but not estrogen stimulate the proliferation of this tumor (unpublished observations). This tumor is, thus, classified as hormone responsive, not hormone dependent. Normal tissues were from mature virgin Balb/cAnNCrlBR mice obtained from Charles River. Animals were maintained and killed according to NIH guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Normal and tumor tissues were dissociated with collagenase (0.05%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (11,13). These cells have been shown in extensive studies to be composed primarily of ductal epithelium with a minor population of myoepithelial cells located basally to the ductal epithelium (14,15). For growth experiments, cell organoids were mixed with neutralized, isosmotic rat collagen, and 0.5 ml containing ~2×10^5 cells pipetted into individual wells of 12-well culture plates containing a preformed bottom layer of collagen gel (0.25 ml) as described (11). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham's F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 µg/ml insulin, 100 U/ml soybean trypsin inhibitor, 1 µg/ml α-tocopherol succinate and other additives as indicated in legends to figures. Cells were grown at 37°C in a 2% CO_2/98% air atmosphere. Medium was changed every other day. When PD 098059 (in dimethyl sulfoxide) was added, it was mixed well by vortexing in fresh medium at the time of medium changes. For determination of cell number, collagen gels containing cells were transferred to 12×75 mm glass tubes, acidified with 1/10 vol 25% acetic acid and incubated at 37°C until the collagen had dissolved. Cells were pelleted, extracted with 70% ethanol overnight and the cell pellets dried for fluorometric DNA assay using diaminobenzoic acid (16). Cell cultures for kinase assays were established as monolayers on collagen-coated culture dishes to facilitate rapid termination. Dishes were coated with collagen by covering the surface of the dish with a thin film of denatured rat tail collagen and air drying overnight. Cell organoids were seeded onto collagen I-coated 10 cm culture dishes in basal F12/DMEM (above) supplemented with 2% porcine serum (for tumor) or 5% BSA (for normal MEC). After 4–6 days, when the cells had grown to near confluence, the cells were rinsed three times with basal serum-free medium and incubated in this medium for 24–36 h prior to the initiation of experimental treatments. Control experiments showed that serum pre-exposure had no effect on the time course of ERK activation for tumor or normal cells.

Termination and preparation of cell extracts

After culture in basal serum-free medium for 24–36 h, the cells were exposed to EGF (20 ng/ml) for various times before termination. Cultures were terminated by aspiration of the culture medium followed by two washes with ice-cold phosphate-buffered saline (PBS) and lysed in 0.5 ml lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerophosphate, 40 mM PNPP, 200 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin. The lysates were mixed by vortexing and clarified by centrifugation (10 min, 13,000 *g*, 4°C). Supernatants were used for immunoprecipitations and western blot analysis. Protein concentration was determined using the BCA assay by Bradford.

MAP kinase assay

MAPK assays were performed using an immune complex kinase filter assay as described previously with minor modifications (17). Briefly, 0.3 mg of sample protein and 10 µl Protein A–Sepharose beads, washed previously in Triton lysis buffer and conjugated with 3 µg anti-MAP kinase rabbit polyclonal antibodies (ERK1 and ERK2), were mixed in a total volume of 1.0 ml at 4°C overnight. Control experiments indicated that this procedure resulted in the immunoprecipitation of >90% of immunoreactive ERK1 and ERK2. The Protein A–Sepharose was then washed twice with 1.0 ml of Triton lysis buffer, twice with 1.0 ml of reaction buffer [50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol] and resuspended in 40 µl of kinase reaction buffer containing 20 µM ATP, 0.25 mg/ml myelin basic protein peptide and 10 µCi [γ-^{32}P]ATP. The samples were incubated for 30 min at 30°C and the reaction was terminated by the addition of 20 µl of 200 mM EDTA, pH 7.0. Samples were centrifuged (13,000 *g*, 2 min, 4°C) and 50 µl of the supernatant was spotted on p81 cation-exchange filter paper (Whatman). The filters were washed three times (8 min each) in 200 ml of 180 mM phosphoric acid, once in 200 ml of 96% ethanol and then air-dried. Radioactivity was quantitated by liquid scintillation counting (Packard).

Western blotting and immunoprecipitation of EGFR

Samples containing 20 µg of total protein were electrophoresed on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBS buffer containing 5% (w/v) dry milk and 0.1% Tween and incubated with anti-phospho-ERK antibody (diluted at 1:3000), ERK1 and ERK2 antibodies (diluted at 1:10,000), or anti-EGF receptor (EGFR) antibody (diluted at 1:2000) as indicated. For immunoprecipitation of the phosphorylated EGFR, 200 µg of total protein was immunoprecipitated overnight with anti-phosphotyrosine antibody (PY20). The immunoprecipitates were resolved on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with EGFR antibody (diluted at 1:1000). For all immunoblotting, a horseradish peroxidase-conjugated secondary antibody was utilized to allow detection of the appropriate bands using enhanced chemiluminescence (Amersham). Immunoprecipitation with antisera to the EGFR followed by immunoblotting with PY20 yielded results similar to the above protocol (data not shown), showing that immunoprecipitation with PY20 precipitated phosphorylated EGFR not just receptor-associated tyrosine phosphorylated phosphoproteins complexed to non-phosphorylated EGFR.

Results

Effect of EGF on proliferation

The concentration dependence and time course for EGF-stimulated growth of normal and mammary tumor cells in serum-free, basal medium containing insulin was examined (Figure 1). Compared with normal mammary epithelial cells in collagen gel culture, the tumor cells showed similar sensitivity to EGF. The relative cell numbers for normal and tumor in Figure 1 show a lower cell number for tumors; however, the magnitude of the EGF-stimulated increase in cell number over basal was similar for tumor and normal cells (5.9- versus 5.1-fold, respectively). The time course of growth for tumors exhibited a rapid phase of growth beginning at 4 days and reaching a plateau at 10 days. This is a typical response pattern in collagen gel culture. Actual increase in cell number is not observed immediately after culture since the cell organoids first have to attach to, and cells begin to migrate into, the collagen gel matrix. In contrast to normal mammary epithelium (18), the proliferative response to EGF is not dependent upon insulin, since EGF was growth-promoting in medium lacking insulin supplementation (not shown).

Effect of EGF on MAP kinase activity

Normal and tumor mammary epithelial cells were cultured on collagen-coated culture plates and exposed to EGF in serum-free medium for different times. Cells cultured in the monolayer system also proliferate in response to EGF over a long time course similar to cells cultured within gels (19). The monolayer system was originally chosen to facilitate rapid termination of cultures. At termination, cells were lysed and total ERK1 and ERK2 activities determined after immunoprecipitation with combined ERK1 and ERK2 antisera. Figure 2A shows that in

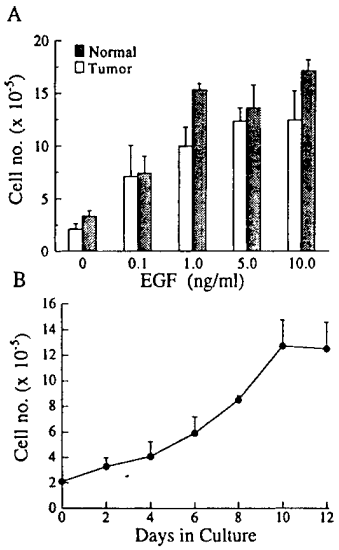

Fig. 1. The effect of EGF on the growth of mammary tumor cells. **(A)** Tumor cells were cultured for 10 days in serum-free medium containing different concentrations of EGF. **(B)** Time course of growth in the presence of EGF (10 ng/ml). Data are expressed as means ± SD of triplicates. Initial cell number was 2.5×10^5. Representative of three experiments.

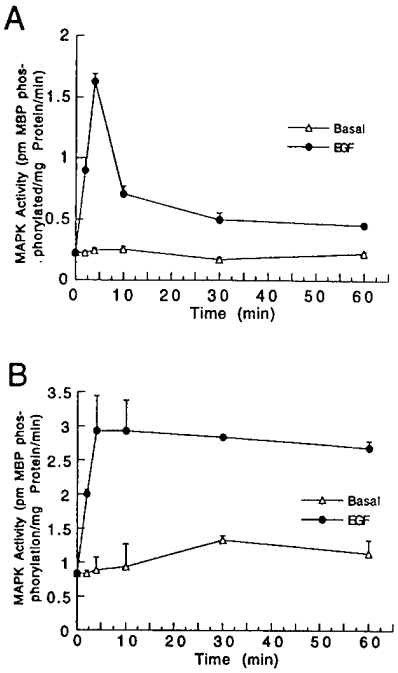

Fig. 2. Time course of MAP kinase activation in response to EGF (10 ng/ml) in **(A)** normal and **(B)** tumor mammary epithelial cells. Cells were plated on collagen-coated culture dishes and grown to near confluence in basal medium containing insulin plus BSA V (2.5 mg/ml) or 2% porcine serum for normal and tumor cells, respectively. Cultures were transferred to insulin-only medium for 24–36 h then treated with or without EGF for the desired time before lysis in extraction buffer. MAP kinase activity was assayed in duplicate in immunoprecipitates of cell extracts as described in the Material and methods. Averages and range of duplicates are plotted. Results are representative of three experiments for normal and tumor cells.

normal cells, ERK activity in immunoprecipitates rose to a peak at 4 min and declined rapidly within 10 min to a level ~2-fold higher than the basal activity. In comparison, tumor cells (Figure 2B) showed a 4-fold higher basal activity and a sustained increase in kinase activity in response to EGF. Although the fold activation by EGF was less than for normal cells (due to the elevated basal activity), the final level of activity was ~2-fold higher. At times >60 min , there was a gradual decline in ERK activation in tumor cells. The difference in the time course for kinase activation (maximum in minutes to 1 h) versus proliferation (maximum after days) reflects the difference in the way the assays are performed. Kinase assays on monolayer cultures are designed to detect an EGF response in a 'synchronized' population i.e. re-exposed to EGF after a period in its absence. Under these conditions, a response to EGF is measurable and illustrative of the effect of EGF. During long term continuous exposure to EGF in proliferation assays, we assume a similar activation is occurring (also accompanied by a prolonged elevated basal level) in dividing cells over time as cells enter the proliferating cell pool (represented by cells at the periphery of colonies or monolayers).

Immunoblot analysis of cell lysates was performed using antisera against phosphorylated and total ERK1 and ERK2 to examine the relative activation of ERK1 and ERK2. Shown in Figure 3 is a comparison of normal (A) and tumor cells (B) cultured in basal medium or stimulated by EGF. For both cell types, ERK1 and ERK2 were activated with no apparent change in total ERKs. Examination of the immunoblotting data suggests that in tumor cells, EGF activates ERK1 (p44) to a greater extent than ERK2 (p42). Densitometry of the ERK bands and comparison of the ratio (ERK1:ERK2) of the fold increases caused by EGF at 4 min showed that this ratio was higher in tumor cell cultures (mean = 5.2, range 2.8–11.8, n = 8) compared with normal cell cultures (mean = 1.35,

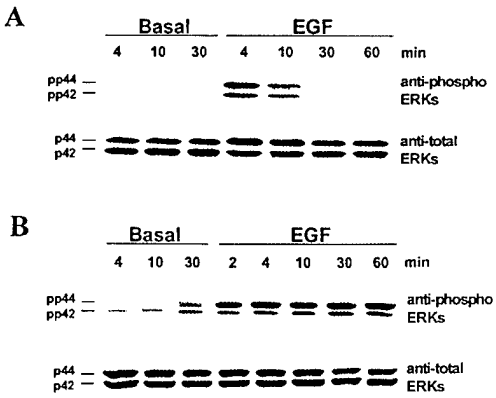

Fig. 3. Western blot analysis of ERK 1 and ERK 2 activity in **(A)** normal and **(B)** tumor mammary epithelial cells. Cell cultures were treated with EGF (10 ng/ml) for 2, 4, 10, 30 and 60 min prior to termination. Basal (B) control cultures were done in parallel. Cell lysates were electrophoresed, transferred to nitrocellulose membranes and blotted with phospho-specific ERK antisera (activated ERKs, top panels) followed by stripping and reprobing with ERK 1 and ERK 2 antisera (total ERKs, lower panels). Results are representative of three experiments for normal and tumor cells.

range 1–2.6, $n = 6$). These results suggest that ERK1 is more important for the EGF response in tumor cells, whereas ERKs 1 and 2 were activated to a similar extent in normal cells.

Recent work has indicated that the total level of MAP kinases is higher in breast cancer compared with normal breast (10). We compared the total level of ERKs in normal and tumor cells by electrophoresing, side-by-side, equal amounts of protein from lysates of these cells cultured in basal medium, and performed immunoblot analyses with antisera to ERK1 and ERK2. ERK bands were subjected to scanning densitometry, summed and normalized to the lowest value. By this analysis, total ERK levels for normal and tumor cells, 1.06 ± 0.19 versus 1.14 ± 0.27 (mean ± SD arbitrary units, $n = 6$), respectively, were found not to be significantly different (Student's t-test, $P > 0.05$). However, the ratio of cytosolic protein to cell number was an average of 73% higher (mean of two experiments) in tumor cells than normal cells, indicating that the basal level of kinases, if normalized to cell number, is higher in tumor cells. Thus, the difference in basal activity between normal and these tumor cells most probably results from differences in the regulation of their state of activation (4-fold) and a smaller contribution from an increased level of kinase per cell.

Dependence of EGF-stimulated cell proliferation on ERK activation

The MEK (ERK kinase) inhibitor, PD 098059, has been shown to specifically and reversibly block ERK activation and inhibit proliferation (20,21). We have shown that EGF, a potent growth stimulant, activates the ERK pathway. To determine if ERK activation by EGF is necessary for the proliferative response to this growth factor, normal and tumor mammary epithelial cells were cultured in the presence of EGF and different concentrations of PD 098059. The effect of PD 090859 on EGF-induced proliferation and ERK activation was then assessed. This compound inhibited the proliferation of tumor and normal cells cultured in the presence of EGF in a concentration-dependent manner (Figure 4A and B). The growth of cells exposed to dimethyl sulfoxide vehicle alone was not affected (not shown). This inhibitory effect is consistent with the interpretation that the ERK pathway is a major signaling pathway involved in EGF mitogenesis in mammary epithelial cells but does not establish that this is the only pathway or that this pathway may not act in synergism with other pathways. That other cellular functions could be affected by PD 098059 was shown by the effect of the inhibitor on the morphology of the colony outgrowths for normal and tumor cells. The typical stellate morphology was reduced and the colonies appeared more rounded as if binding and spreading of cells into the collagen matrix was inhibited. This morphological change was observed from the initiation of the cultures (within 2 days when attachment to the gel becomes observable) for both normal and tumor cells, the effect as observed in tumor cells is illustrated in Figure 5. This change in morphogenesis indicates that the ERK pathway is involved in the initiation and/or maintenance of matrix interactions, perhaps in the generation of adhesion plaques or the regulation of the cytoskeleton. Since PD 098059 has been shown to reversibly inhibit MEK, we performed experiments in which tumor cells were cultured in the presence of EGF and PD 098059 for the first 6 days *in vitro* then switched to inhibitor-free medium. After 6 days, growth was inhibited by 33% in cultures containing PD 098059, and upon removal of the inhibitor, cell number

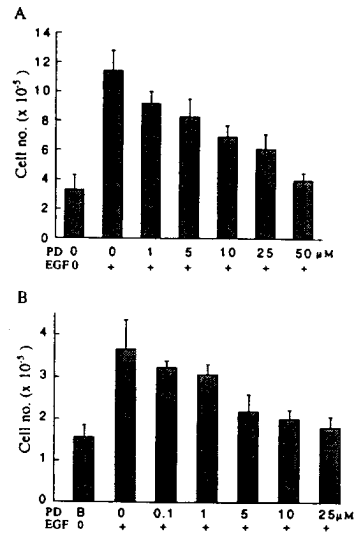

Fig. 4. PD 098059 (PD) inhibition of EGF-stimulated growth. (A) Mammary tumor (means ± SD of triplicates, representative of three experiments) or (B) normal mammary epithelial cells (means ± SE, $n = 3$), were cultured for 10 days in medium containing insulin and EGF (10 ng/ml) without or with PD (0.1–50 μM) added at the initiation of the cultures.

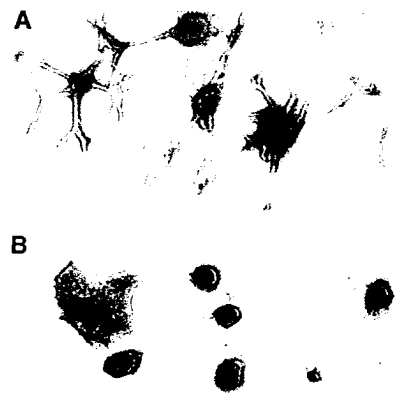

Fig. 5. Morphology of mammary tumor epithelial cell colonies in medium containing (A) EGF (10 ng/ml) or (B) EGF plus PD 098059 (50 μM). Phase photographs were taken after 10 days of culture. Magnification, ×616.

increased to a level similar to cells cultured continuously in the presence of only EGF (Figure 6). The effect of the inhibitor on colony morphology was also reversible with the growing colonies assuming a more stellate morphology. The continuous presence of EGF was required for reversibility of the growth inhibition. This reversibility suggests that restoration of ERK activation by EGF is sufficient to promote proliferation and reestablish matrix interactions, phenomena that may be related.

To confirm that PD 098059 was inhibiting ERK activity, cells were cultured in the presence and absence of this compound and ERK activation was assessed by electrophoresis

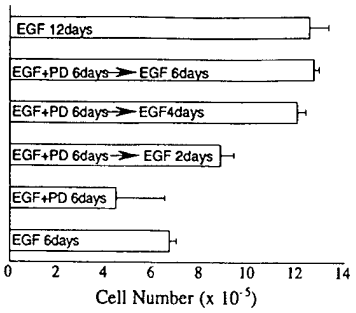

Fig. 6. Reversibility of PD 098059 growth inhibition in tumor cells. PD was added to EGF-stimulated cultures for 6 days (EGF + PD 6 days). At this time, parallel cultures in PD were switched to EGF-only medium and cultured for an additional 2, 4 or 6 days before termination. Control cultures containing only EGF were terminated at 6 days (EGF 6 day) and 12 days (EGF 12 days). Similar results were obtained in a second experiment.

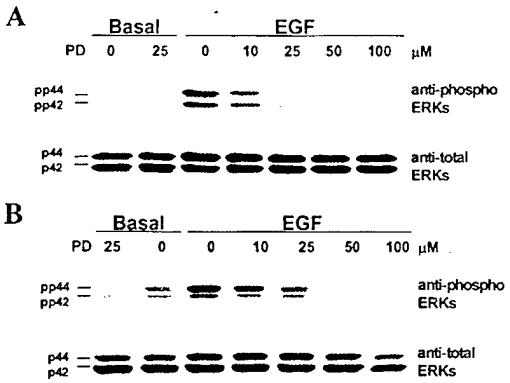

Fig. 7. Effect of PD 098059 on MAP kinase activation in (A) normal and (B) tumor mammary epithelial cells. Cells were preincubated in inhibitor for 1 h before EGF was added. After 4 min in EGF, cultures were terminated and western blotting for phosphorylated and total ERKs was performed as described in Figure 3. Representative of three experiments.

and immunoblotting of cell lysates with antisera to phosphorylated ERK1 and ERK2. Figure 7 shows that ERK phosphorylation was inhibited in normal and tumor cells in a concentration dependent manner with complete inhibition of EGF-stimulated ERK phosphorylation at concentrations (50–100 μM).

EGF receptor phosphorylation in normal and tumor cells

As shown in Figure 1, maximum EGF-stimulated ERK activation was prolonged in the tumor and relatively transient in normal cells. EGF binding to its receptor results in receptor dimerization and transphosphorylation of tyrosines by the receptor kinases (4). We monitored tyrosine phosphorylation of the receptor to compare the time course of receptor activation to ERK activation. Figure 8 shows that the time course of EGF-induced receptor autophosphorylation differed between normal and tumor cells. Receptor phosphorylation was sustained in tumor but not normal cells where it declined significantly by 30 min. Thus, we find a correlation between ligand-induced EGF receptor activation and ERK activation, suggesting that the extended time course of activation in tumor cells is due to continued stimulation of the ERK pathway

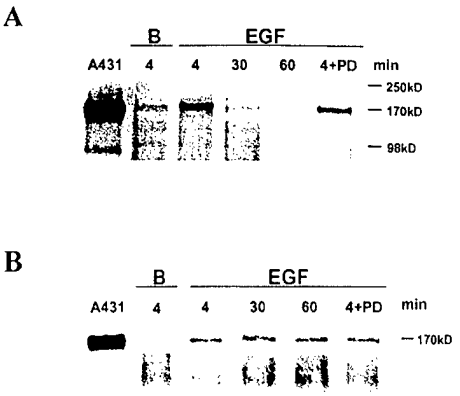

Fig. 8. Phosphorylation of the EGF receptor in response to EGF and PD 098059. (A) Normal and (B) tumor mammary epithelial cell cultures were stimulated by EGF for 4–60 min. PD 098059 was also included in parallel for cells exposed to EGF for 4 min (peak response). Cell lysates were immunoprecipitated with anti-phosphotyrosine antisera then the immunoprecipitates were subjected to western blot analysis with EGF receptor antisera. The 170 kDa band is the phosphorylated EGF receptor. A431 cell lysates were run as a positive control. Representative of three experiments. B, basal untreated culture.

initiated at the EGF receptor. Figure 8 also shows that PD 098059 pretreatment of cells did not block EGF receptor autophosphorylation [or EGF-induced c-jun kinase (JNK1 and JNK2) activation (unpublished observation)], in agreement with the reported specificity of this agent. Western immunoblotting of cell lysates with antisera to the EGF receptor showed that there was no apparent change in total receptor level during a 1 h time course in normal and tumor cells (data not shown).

Discussion

These studies were initiated to examine the hypothesis that the regulation of the activity of the MAP kinases, ERK1 and ERK2 could be altered during mammary tumorigenesis. This hypothesis was tested by comparing the effect of EGF, a known activator of this pathway, on proliferation and activation of ERKs in normal and tumor mammary epithelial cells. Previous work examining the effect of growth factor stimulation of confluent and quiescent cultures of fibroblasts has shown that after an early peak in ERK activation, there is a slower elevation in activity over several hours to a level above basal. This later rise or sustained increase in activity is associated with the stimulation of DNA synthesis (22–25). The time course of ERK activation in our primary epithelial cell cultures differs from that observed in fibroblasts by lacking this biphasic quality. In normal mammary epithelium, EGF stimulates peak ERK activity at 4–5 min post-exposure, with a decline to a steady level ~2-fold above basal by 30 min; this is presumably sufficient and necessary to maintain mitogenesis as revealed using the upstream MEK inhibitor, PD 098059. Thus, during proliferation occurring over a period of 7–10 days, the continuous presence of EGF would stimulate ERK activity in the proliferating pool of cells shown to be localized at the periphery of the colonies or tips of growing projections (26).

In the tumor cells, EGF stimulation caused ERK activity to rise to a plateau at 4–5 min before declining slowly but remaining elevated ~3-fold above the basal level. A preferential activation of ERK1 by EGF was also observed.

Both enzymes appear to be present in approximately equal levels as observed by immunoblotting, suggesting that MEK is preferentially phosphorylating ERK1 or that the dephosphorylation of these proteins is differentially regulated in the tumor cells. Both ERKs phosphorylate the same sequence in peptide substrates but may differ in their nuclear translocation or association with cell surface receptors (27). In fact, nuclear translocation may be associated with sustained ERK activation observed in fibroblasts or PC12 cells that is associated with mitogenesis or differentiation, respectively (28,29). We can hypothesize that sustained activation and nuclear localization of ERK1 in tumor cells may play a role in tumorigenesis.

In spite of the enhanced stimulation of ERK activity by EGF, we observed no dramatic enhancement of EGF mitogenesis *in vitro*. This could reflect a growth limitation of the *in vitro* system in which proliferation plateaus at 10–14 days, or suggest that EGF also affects non-proliferative signaling pathways affecting tumor cell invasion, differentiation or cell–cell interactions. EGF may also affect proliferation indirectly *in vivo* by modulating responses to other growth-stimulatory factors such as hormones. Estrogenic control of proliferation has been linked to EGF (30–32) and recently, an estrogen non-responsive breast cancer cell line was found to possess an elevated basal MAP kinase activity (33). Below, we speculate on possible roles that EGF may play in regulating matrix interactions, especially the regulation of matrix-responsive signaling pathways that may play a role in tumor progression.

For both cell types, we observed a correlation between tyrosine phosphorylation of the EGF receptor and ERK activation. Thus, the difference in the time course of EGF-induced ERK activation between normal and tumor cells was due to the a sustained activation of the EGF receptor, not a direct modulation of ERK activity downstream of MEK that is independent of EGF receptor activation. This response is reminiscent of a mutant EGFR found in human cancers, which exhibits a low level of constitutive activity combined with a decrease in down regulation resulting in an amplification of signaling and possibly tumorigenesis (34). The prolonged EGFR activation could be explained by attenuated down regulation or an alteration in the regulation of its phosphorylation state by other kinases or phosphatases. For example, EGFR kinase activity is inhibited by phosphotyrosine phosphatases regulated by protein kinase C acting through the MAP kinase pathway (35,36), arachidonic acid metabolites can augment the mitogenic effect of EGF by enhancing the phosphorylation of the EGF receptor (37), and phosphatases (PTP1C) have been found associated with the EGFR that can dephosphorylate the receptor upon co-stimulation of cells by ligands to G protein-coupled receptors (38). Also, treatment of primary cultures of mammary epithelium with the tyrosine phosphatase inhibitor, pervanadate, could potentiate cell proliferation stimulated by a suboptimal concentration of EGF that itself is not mitogenic (39).

Another key difference between normal and tumor cells was an elevation in the level of basal kinase activity. We could not reproducibly detect any difference in basal EGFR phosphorylation between normal and tumor cells, although, in breast cancer cells overexpressing ErbB-2, an elevation in ligand-independent basal activity was observed (6). This tumor does not proliferate in the absence of EGF or other mitogens, rendering autocrine stimulation an unlikely explanation for the elevation in ERK activity in basal medium.

It is possible that altered cell matrix interactions seen as changes in tumor morphology *in vivo* can affect basal ERK activity. Many studies have shown that membrane receptors for matrix molecules (integrins) can signal via the ERK pathway, illustrating how proliferation control can be linked to matrix interactions (40–43). Cell matrix interactions can be EGF dependent as well. We have also observed an effect of PD 098059 on the morphology of the outgrowths in collagen gels. While not directly showing an effect on matrix binding, we speculate that this result indicates that the ERK pathway is required for proper matrix interaction and possibly also cell migration. The synthesis and deposition of matrix molecules (Type IV collagen, laminin, fibronectin) is regulated by substrate (44) and growth factors such as EGF (45). EGF then can therefore affect (directly or in cooperation with other pathways) proliferation and morphogenesis by ERK-directed events that establish matrix interactions such as adhesion complexes and then act as positive or negative co-regulators of matrix-dependent signaling. As an example, overexpression of the EGFR has been associated with a defect in integrin function in human cutaneous squamous carcinoma cells (46).

Another possible explanation for an elevation in basal activity is a change in the regulation of the expression or activity of ERK specific threonin/tyrosine phosphatases that inactivate ERK (24,47,48). Recently, it has been shown in Rat-1 cells that the expression of the ERK phosphatase MKP-1 is controlled by growth factors acting via ERK- and calcium-dependent pathways. Interestingly, treatment with sodium orthovanadate, a phosphatase inhibitor, elevated basal ERK activity in the absence of growth factor (24). Thus, an attenuation in the expression or activation of phosphatases might be manifest as a rise in basal ERK activity.

In conclusion, we find that a hormone-responsive mammary tumor exhibits multiple alterations in the regulation of the activity of the MAP kinases, ERK1 and ERK2 with a smaller change in enzyme level. These alterations are related in part to a change in the regulation of the EGFR but also involve pathways that are independent of EGF. These kinds of alterations in a pathway associated with proliferation and estrogen receptor regulation may play a role in tumor progression and the evolution of hormone independence in breast cancer.

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