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13. ABSTRACT *(Maximum 200*

The purpose of this project is to better understand the signal transduction pathways that mediate growth inhibition of breast cancer cells. In fibroblasts, the classical MAPK pathway (Ras/Raf-1/MEK/ERK) mediates the proliferative response by growth factors; while the novel MAPK pathway (Ras/Rac/MEKK1/JNKK1/JNK) may be involved in growth inhibition. A growth factor receptor ErbB-2, which is a critical regulator of the growth and differentiation of breast cells, is overexpressed in 25% of human mammary tumors. Interestingly, its ligand NDF (neu differentiation factor) can either induce the proliferation or differentiation of breast cancer cells. Using mammary carcinoma cells, which proliferate or differentiate upon NDF addition, the respective roles of the two MAPK cascades in controlling cell growth and differentiation are examined. As demonstrated in this report, both the JNK and the ERK cascades are activated at similar kinetics in the differentiative AU565 and the proliferative SKBR3 cells. However, JNK is marginally activated in the proliferative MCF7 cells and not activated in the differentiative MDAMB453 cells. In contrast, ERK is strongly activated by NDF in MCF7 cells, while it is only moderately activated in MDAMB453 cells. Thus, no correlation has been found between the activation profile of the JNK and ERK cascades and the growth responses of the above four mammary carcinoma cells.

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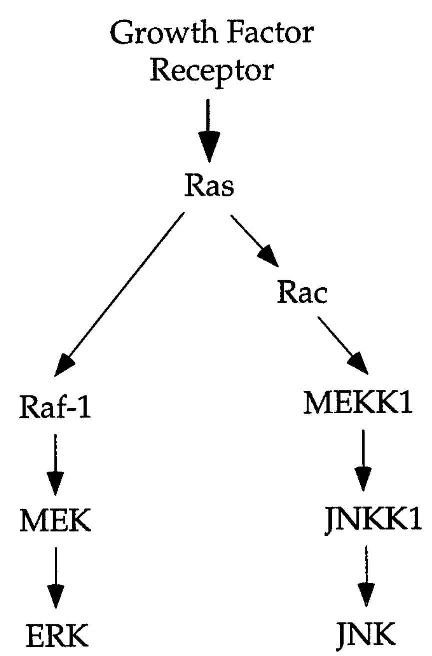
Table of Contents

INTRODUCTION

The focus of this report is to investigate the respective roles of different MAPK cascades in mediating distinct cellular responses to neu differentiation factor (NDF) in breast cancer cells. Specifically, whether the proliferative or the differentiative response to NDF in breast cancer cells is due to differential activation of the Raf-1/ERK or the MEKK1/JNK MAPK cascades.

In mammalian cells, the classical MAPK (mitogen-activated protein kinase or extracellular signal-regulated kinase, ERK) pathway (Ras/Raf-1/MEK/ERK) is an important mediator of the proliferative response in fibroblasts signaled by growth factors, although it has been reported that it could also induce the differentiation of certain cell types, such as PC12 pheochromocytoma cells (1-4). The ERKs are phosphorylated and activated by the MEKs (5). The MEKs in turn are phosphorylated and activated by Raf-1, which itself is activated by growth factor receptors via Ha-Ras (6-9). Recently, two novel MAPKs, JNK1 and JNK2 (Jun amino-terminal kinase or stress-activated protein kinase, SAPK) were identified and cloned (10-13). Instead of the MEKs, the JNKs are phosphorylated and activated by JNK kinases, one of which was molecularly cloned (JNKK1 or SEK1 or MKK4) (14-16). JNKK1, in turn, is not activated by Raf-1 but by another protein kinase called MEKK1 (15, 17). Although activation of MEKK1 is also Ras-dependent (18, 19), it does not occur through direct interaction. Recently, the small GTP binding protein Rac, a member of the Ras superfamily, was shown to act between Ras and MEKK1 (20). As Rac binds to the protein kinase PAK1 in a GTP-dependent manner and stimulates its autophosphorylation activity (21), PAK1 or a related kinase may mediate its effect on the JNK pathway through direct phosphorylation of MEKK1. Rac does not activate the ERK pathway (20). Therefore, Ras/Rac/MEKK1/JNKK1/JNK forms a novel MAPK pathway, independent of and separate from the classical Ras/Raf-1/MEK/ERK MAPK cascade. A schematic representation of the two signal transduction pathways initiated at growth factor receptors is shown.



The distinct protein kinase constituents and substrate specificities of the two MAPK pathways suggest that they may have different biological roles and mediate distinct cellular responses (2). In addition to growth factors, the JNKs are also activated by a variety of stress signals, including UV irradiation, DNA damaging agents and tumor necrosis factor α (TNFα), which cause growth arrest or apoptosis rather than cell proliferation (10, 11, 12, 13, 15, 19). The pathways by which these stimuli activate the JNKs are not yet understood, and unlike the growth factor response, these pathways do not involve Ras or Rac (20). Recently, a proteolytic cascade has been implicated in apoptosis (22). However, it is not ruled out that MAPK cascades are also involved in transducing the death signal. Indeed, preliminary results suggest that selective activation of the MEKK1/JNK pathway inhibits PC12 cell proliferation and triggers apoptosis in NIH3T3 fibroblasts. In direct contrast, the ERKs are mainly activated by growth factors and in most cell types exhibit a very weak response to stress signals (13). Constitutive activation of the classical ERK cascade leads to cell transformation and, in some cases, to cell differentiation (1). Although activation of the JNK cascade may play an auxiliary role in such responses, recent results suggest that when activated without concomitant activation of the ERK cascade, the JNK cascade may transduce growth inhibitory signals and may even lead to cell death (23). Based on these results, it is tempting to speculate that preferential Raf-1/ERK activation results in cell proliferation; whereas preferential MEKK1/JNK activation may cause growth arrest, or even lead to induction of apoptosis in certain cells.

This proposal is therefore focused on the roles of the different MAPK cascades in mediating distinct growth responses and how the balance between these pathways maintains normal cell growth. Breast cancer cells offer an excellent model system for such studies. Approximately 25% of primary breast tumors overexpress the ErbB-2 protein, which is a close relative of the EGF receptor (EGFR) (24). Importantly, the activity of ErbB-2 directly correlates with clinical prognosis, and its inhibition results in reversion of mammary carcinomas (24, 25). Recently, a ligand involved in its activation called neu differentiation factor (NDF, or heregulin, HRG) was identified (26, 27). Interestingly, NDF appears to have dual growth regulatory properties. In some mammary carcinoma cell lines (e.g., AU-565 and MDA-MB453), activation of ErbB-2 by NDF blocks cell proliferation, and induces cell differentiation and growth arrest (24, 26). However, in other breast cancer cell lines (e.g., SKBR-3 and MCF-7), NDF stimulates cell proliferation (24, 27). Furthermore, NDF activates ErbB-2 only in mammary tumor cells but not in ovarian carcinomas or transfected fibroblasts, suggesting that auxiliary proteins are required for ErbB-2 activation (28). Recently, additional members of the ErbB family, ErbB-3 and ErbB-4, were identified to be the direct receptors for NDF (28). Binding of NDF to ErbB-3 or ErbB-4 induces heterodimerization with ErbB-2, followed by its phosphorylation and activation (28). Like EGFR, ErbB-3 can cooperate with ErbB-2 in neoplastic transformation (24, 28a). However, the biological function of ErbB-2/ErbB-4 heterodimer is not known. Therefore, it will be interesting to propose that activation of ErbB-2/ErbB-3 or ErbB-2/EGFR heterodimer signals a mitogenic response, whereas activation of ErbB-2/ErbB-4 heterodimer may be responsible for NDF-induced cell differentiation. Since all of the ErbB proteins are quite similar in their cytoplasmic domains (28), it is not clear how the activation of different heterodimers elicits distinct biological responses.

BODY

Assumptions:

The signaling pathways that mediate cellular responses to ErbB-2 activation are not well understood, but based on its similarity to the EGF receptor, are likely to involve ERK and JNK activation. It is tempting to speculate that preferential Raf-1/ERK activation results in cell proliferation; whereas preferential MEKK1/JNK activation may cause growth arrest, or even lead to induction of apoptosis. It is therefore of interest to examine whether the proliferative or the differentiative response to NDF in different breast cancer cells is due to differential activation of the ERK or the JNK cascades, respectively. Using mammary carcinoma cells, which either proliferate or differentiate in response to NDF, the respective roles of the Raf-1/ERK and the MEKK1/JNK pathways in the response to NDF could be examined. If the hypothesis is correct, I will expect to see that upon NDF binding, the Raf-1/ERK cascade will be preferentially activated in proliferation-response cells (SKBR-3 and MCF-7), whereas the MEKK1/JNK cascade will be preferentially activated in differentiation-response cells (AU-565 and MDA-MB453).

Experimental Methods and Procedures:

To address the above question, human mammary carcinoma cell lines SKBR-3 and MCF-7, which proliferate in response to NDF (24, 27); and AU-565 and MDA-MB453, which differentiate upon NDF binding (24, 26), will be used. Various NDF isoforms have been cloned (27, 29a). As the different isoforms don't seem to differ in their biological activities and the β isoforms display higher receptor binding affinities than the α isoforms (27, 29a), NDFβ1 will be used for the following studies. NDF, whenever mentioned below, will refer to NDFβ1.

Cells will be incubated in the presence or absence of 0.2 nM or 1 nM NDF for 5 or 15 min (26), after which whole cell extracts will be prepared. Activation of ERK and JNK will be examined by immune complex kinase assay using myelin basic protein (MBP) or GST-cJun (1-79) as substrates, respectively (19). Specifically, ERK2 will be immunoprecipitated by an anti-ERK2 antibody, followed by incubation with MBP in kinase buffer containing [γ-^{32}P]ATP at 30°C for 25 min. In parallel, JNK will be immunoprecipitated by an anti-JNK antibody, followed by incubation with GST-cJun (1-79) in the presence of [γ-^{32}P]ATP at 30°C for 25 min. Phosphorylated proteins will be separated by SDS-PAGE and visualized by autoradiography. Untreated cells will serve as a negative control to determine background kinase activities. Cells treated with EGF (100 ng/ml) will be used as a positive control for ERK activation, and cells exposed to UV irradiation (40 J/m^2 for 20 sec) will be used as a positive control for JNK activation. If the hypothesis is correct, I will expect to see that upon NDF binding, the Raf-1/ERK cascade will be preferentially activated in the proliferation-response cells (SKBR-3 and MCF-7), whereas the MEKK1/JNK cascade will be preferentially activated in the differentiation-response cells (AU-565 and MDA-MB453).

Results and Discussion:

As shown in the attached figures, both the JNK and the ERK cascades are activated at similar kinetics in the differentiative AU-565 and the proliferative SKBR-3 cells. However, JNK is marginally activated in the proliferative MCF-7 cells and not activated in the differentiative MDA-MB453 cells. In contrast, ERK is strongly activated by NDF in MCF-7 cells, while it is only moderately activated in MDA-MB453 cells. Thus, no correlation has been found between the activation profiles of the JNK and ERK cascades and the growth responses of the above four mammary carcinoma cells. The role of JNK and ERK signal transduction pathways in the growth control of breast cancer cells is not clear at this point, which requires further investigation. Only task 1 in the statement of work has been completed, the remaining objectives may help address the contribution of the JNK and ERK cascades in the proliferation and differentiation of mammary carcinoma cells.

Figure Legends

Figure 1. Time course and dose response of NDF-induced JNK activation in AU565 and SKBR3 cells. Cells were incubated in the presence or absence of 0.2 nM or 1 nM NDF for 5 or 15 min. After cell lysis, JNK activity was examined by immune complex kinase assay using GST-cJun (1-79) as substrate. JNK immunoprecipitated from UV irradiated or EGF treated cells was used as controls.

Figure 2. Time course and dose response of NDF-induced ERK activation in AU565 and SKBR3 cells. Cells were incubated in the presence or absence of 0.2 nM or 1 nM NDF for 5 or 15 min. After cell lysis, ERK activity was examined by immune complex kinase assay using MBP as substrate. ERK immunoprecipitated from UV irradiated or EGF treated cells was used as controls.

Figure 3. Time course and dose response of NDF-induced JNK activation in MCF7 and MDAMB453 cells. JNK activity was examined as described in Figure 1 legend except that MCF7 and MDAMB453 cells were used.

Figure 4. Time course and dose response of NDF-induced ERK activation in MCF7 and MDAMB453 cells. ERK activity was examined as described in Figure 2 legend except that MCF7 and MDAMB453 cells were used.

Time Course and Dose Response of NDF-induced JNK Activation in AU565 and SKBR3 Cells

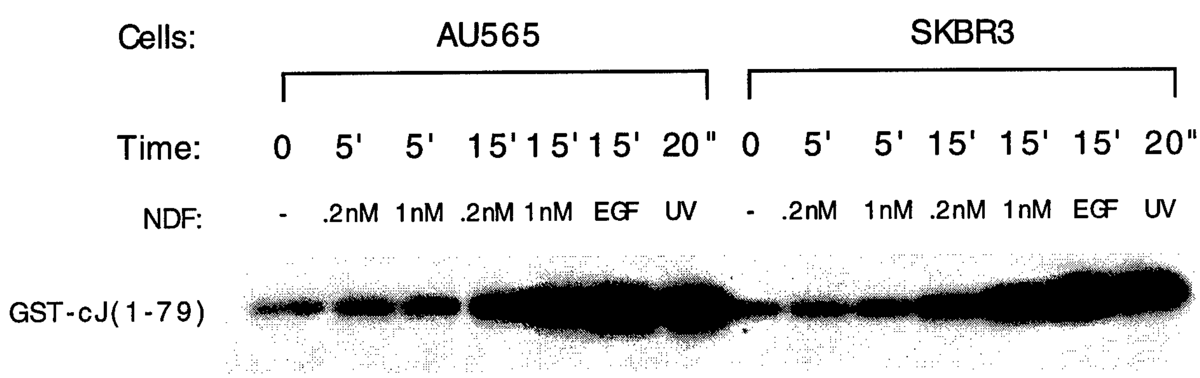


Figure 1

Time Course and Dose Response of NDF-induced ERK Activation in AU565 and SKBR3 Cells

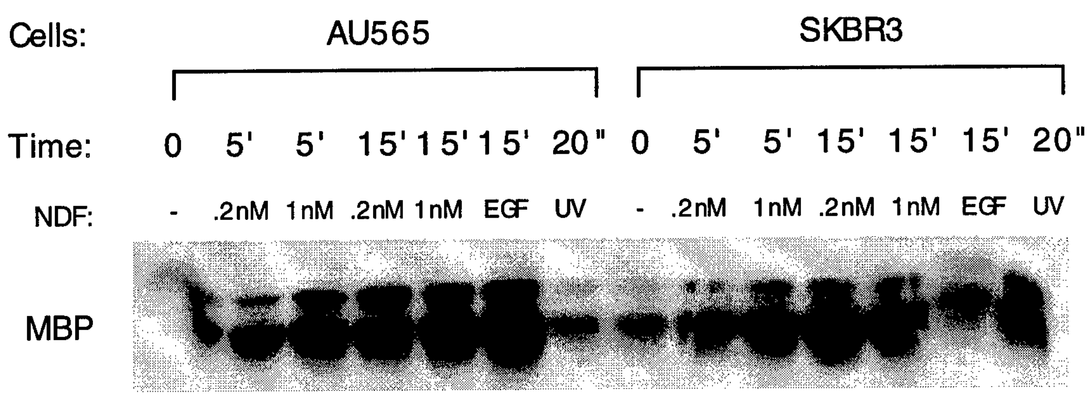


Figure 2

Time Course and Dose Response of NDF-induced JNK Activation in MCF7 and MDAMB453 Cells

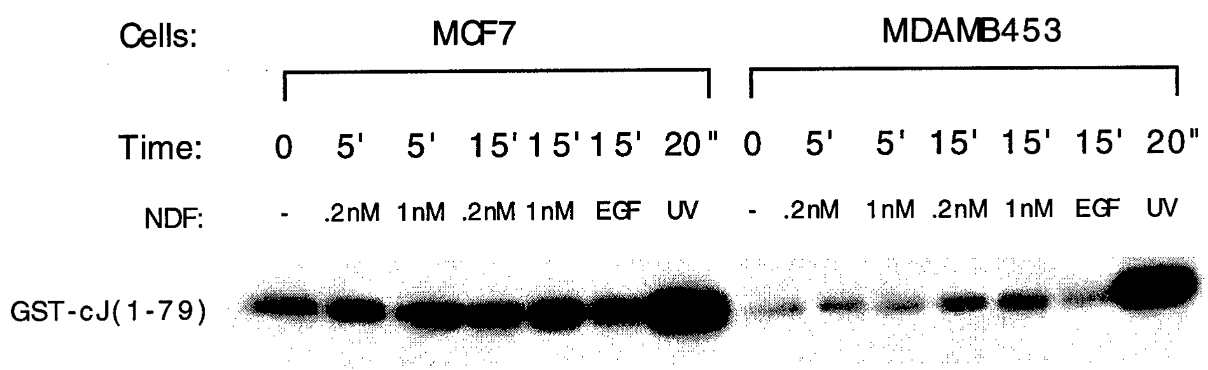


Figure 3

Time Course and Dose Response of NDF-induced ERK Activation in MCF7 and MDAMB453 Cells

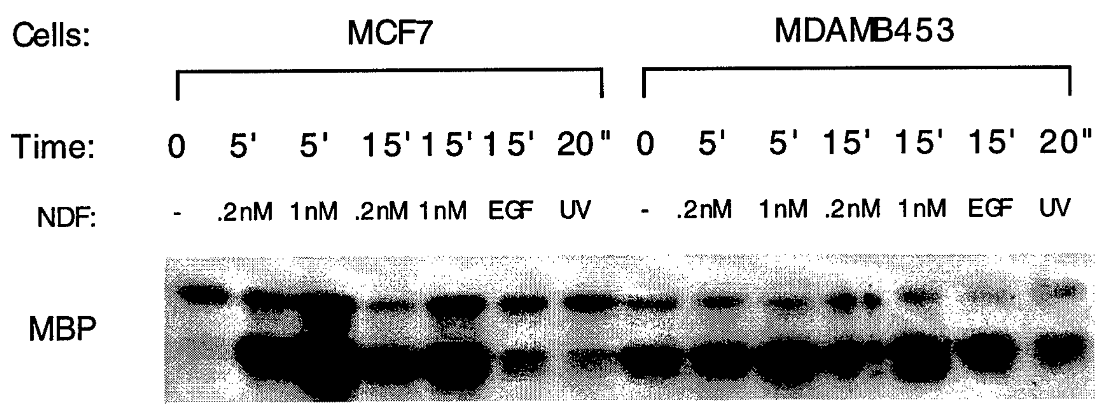


Figure 4

CONCLUSIONS

As no correlation was found between the activation profiles of the JNK and ERK cascades and the growth responses of the above four mammary carcinoma cells, the activation of JNK and ERK in response to NDF in the above four mammary carcinoma cells seems to be cell line dependent. However, selective inhibition of either pathway through generation of stable cell lines or small molecule inhibitors, as specified in tasks 3-5 in the statement of work will help clarify this issue.

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