Signal transduction via mitogen-activated protein kinase pathways plays a key role in a variety of cellular responses, including cell proliferation, differentiation, tumor promotion, and cell death. c-Jun N-terminal kinases (JNKs) are identified as members of the mitogen-activated protein kinase family and are known to phosphorylate and activate several transcription factors, including c-Jun, ATF, and Elk-1. However, the role of JNK activation in tumor promotion is not yet defined. Because previous studies have indicated that exposure of JB6 Cl 41 cells to either 12-O-tetradecanoylphorbol-13-acetate (TPA) or tumor necrosis factor-α (TNF-α) results in cell transformation, we investigated the role of JNKs in this biological process by using dominant negative JNK, and the cell transformation model JB6 Cl 41 cells. Incubation of Cl 41 cells with TNF-α led to cell transformation and activation of JNKs. Introduction of the dominant negative mutant of JNK1 into JB6 Cl 41 cells specifically inhibited TNF-α-induced activation of JNKs, but not Erks and p38 kinases. Most importantly, expressing dominant negative mutant JNK inhibited TNF-α-induced cell transformation but not TPA-induced cell transformation. Our results directly demonstrated for the first time that JNK activation is required for TNF-α but not TPA-induced cell transformation.

The mitogen-activated protein kinase (MAPK) families are protein serine/threonine kinases that are rapidly activated upon extracellular stimulation (1–5). These kinase families include extracellular signal-regulated kinases (Erks), c-Jun N-terminal kinases (JNKs), and p38 kinases (2, 6). The first cloned and characterized MAP kinase cascade was the pathway leading to the activation of Erks (7). The mammalian Erks are involved in growth factor-mediated activation and differentiation of a variety of cells and are activated by growth factors and mitogens, such as epidermal growth factor (EGF), insulin, and phorbol esters (8–10). The pathway leading to Erk activation is through phosphorylation of both threonine and tyrosine residues of Erks by its upstream protein kinase known as MEK (9, 11–13). MEK itself is activated by phosphorylation on two conserved serine residues by several distinct serine/threonine kinases, including Raf, Mos, and MEK kinase 1 (MEKK1) (14–17). The function of Erks has been well defined and linked to mitogenic stimulation, cell differentiation, and transformation (8, 10, 18). For the last five years, a great deal of attention has been given to the characterization of the signaling pathways that lead to JNK activation (3, 6, 15, 19–21). JNKs are activated by a variety of different types of cellular stresses as well as extracellular stimuli, such as UV irradiation, tumor necrosis factor-α (TNF-α), and expression of the transforming oncogene (6, 20, 22, 23). The JNK subgroup includes the products of three related genes, JNK1 (6), JNK2 (5, 24), and JNK3 (25). Whereas JNK1 and JNK2 are expressed in most cells (5), JNK3 appears to be limited to expression in neuronal cells (25). The signaling pathway leading to JNK activation involves Rac, MEKK1, and JNK kinase 1 (26–29). It was reported that both Rac and cdc42Hs activate JNKs but not Erks (3, 30). A dominant negative inhibitory mutant of Rac blocks JNK activation by Ha-Ras and v-Src (3, 30). Overexpression of dominant negative Rac can inhibit JNK activation by TNF-α and IL-1 (3), whereas a dominant negative Ha-Ras mutant does not block TNF-α- or IL-1-induced JNK activation (31, 32). Furthermore, it was demonstrated that UV irradiation can activate JNKs in the absence of JNK kinase (33), suggesting that activation of JNKs by UV irradiation may be through a pathway that differs from the pathway described previously (33). Although we learned a great deal about the pathways of JNKs, little is known about their biological function, especially in tumor promotion. Some studies indicated that JNK activation is responsible for triggering apoptosis in response to different chemical agents (34, 35). JNK activation is also thought to be involved in the induction of cyclooxygenase 2, which plays an important role in the inflammatory responses by catalyzing the production of prostaglandins (36). There is indirect evidence that JNKs may be involved in cell proliferation and tumorigenesis (23). However, the potential role for the JNK pathway in cell transformation is still unclear. It is therefore essential to determine directly whether activation of JNK plays a critical role in tumor promotion. Previous studies have demonstrated that in a mouse epidermal JB6 cell line, different tumor promoters, such as TPA, EGF, and TNF-α, induce the formation of large, tumorigenic anchorage-independent colonies in soft agar at a high frequency (21, 37, 38). Therefore, this model and a well characterized dominant negative JNK mutant (APF) were used to directly investigate the role of JNKs in tumor promoter-induced cell transformation.

MATERIALS AND METHODS

Plasmids and Reagents—CMV-neo vector plasmid was constructed as previously reported (38, 39); dominant negative JNK (pCDNA-flag-JNK, (APF)) was from Dr. Roger J. Davis, Department of Biochemistry.
and Molecular Biology, University of Massachusetts Medical School (6, 40). The dominant negative JNK mutant (APF) is the double point mutation that changes the phosphorylation sites Thr183 and Tyr185 to Ala and Phe, respectively (6, 40). Fetal bovine serum (FBS) and Eagle’s minimal essential medium (MEM) were from BioWhittaker; LipofectAMINE was from Life Technologies Inc.; and TPA was from Sigma. Rabbit polyclonal IgG against protein kinase C α was from Santa Cruz Biotechnology. EGF was from Collaborative Research; human TNF-α was purchased from Roche Molecular Biochemicals; and PhosphoPlus MAPK antibody kit was from New England Biolabs.

Cell Culture—JB6 P+ mouse epidermal cell line, Cl 41, and dominant negative JNK, (pcDNA-flag-JNK, (APF)) plasmid DNA and 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced by 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and cell suspensions were plated into 75 ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfected Cl 41 CMV-neo mass1 and Cl 41 DN JNK1 mass2, were cultured in monolayers at 37 °C, 5% CO2, using MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin/ml (38, 39).

Generation of Stable Cotransfectants—JB6 Cl 41 cells were cultured in a 6-well plate until they reached 85–90% confluence. We used 1 μg of CMV-neo vector with or without 12 μg of dominant negative JNK, (pcDNA-flag-JNK, (APF)) plasmid DNA and 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced by 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and cell suspensions were plated into 75 ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfected Cl 41 CMV-neo mass1 and Cl 41 DN JNK1 mass2, were cultured in monolayers at 37 °C, 5% CO2, using MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin/ml (38, 39).

Phosphorylation Analysis for Erks, JNKs, and p38 Kinase—Immunoblot for phosphorylated proteins of Erks, JNKs, and p38 kinase was carried out using phospho-specific MAPK antibodies against phosphorylated sites of Erks, JNKs, or p38 kinase (41). Antibodies from New England Biolabs were used according to the manufacturer’s recommendations. Antibody-bound proteins were detected by chemiluminescence (ECL, New England Biolabs).

Anchorage-independent Transformation Assay—1 × 104 cells were exposed to TPA- and TNF-α in BME agar containing 15% FBS. The cultures were maintained in a 37 °C, 5% CO2 incubator for 2 weeks. The TPA- and TNF-α-induced cell colonies were scored at 14 days after cells were exposed to TPA or TNF-α.

RESULTS

Induction of Cell Transformation by TNF-α—TNF-α plays an important role in the pathogenesis of many diseases and elicits a wide range of biological responses, including cell proliferation, differentiation, and apoptosis, depending on the cell type and its state of differentiation (42–45). We have investigated these effects on signal transduction pathways, we also studied these effects on MAPK antibody kit was from New England Biolabs.

Expression of Dominant Negative JNK, (pcDNA-flag-JNK, (APF)) plasmid DNA and 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced by 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and cell suspensions were plated into 75 ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfected Cl 41 CMV-neo mass1 and Cl 41 DN JNK1 mass2, were cultured in monolayers at 37 °C, 5% CO2, using MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin/ml (38, 39).

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Anchorage-independent Transformation Assay—1 × 104 cells were exposed to TPA- and TNF-α in BME agar containing 15% FBS. The cultures were maintained in a 37 °C, 5% CO2 incubator for 2 weeks. The TPA- and TNF-α-induced cell colonies were scored at 14 days after cells were exposed to TPA or TNF-α.

RESULTS

Induction of Cell Transformation by TNF-α—TNF-α plays an important role in the pathogenesis of many diseases and elicits a wide range of biological responses, including cell proliferation, differentiation, and apoptosis, depending on the cell type and its state of differentiation (42–45). We have investigated the tumor promotion activity of TNF-α in JB6 Cl 41 cells. The results show that exposure of Cl 41 cells to either TPA or TNF-α causes the anchorage-independent growth of Cl 41 cells (Fig. 1). The transformation rate of TNF-α is similar to that of TPA, a widely used tumor promoter (Fig. 1).

TNF-α Induces the Activation of JNKs, Erks, and p38 Kinases—Previously, we and others reported that signal transduction pathways leading to activated protein-1 activation are required for tumor promoter (TPA, EGF, or TNF-α)-induced cell transformation in a JB6 cell model (8, 38, 46). It is known that activated protein-1 activation is through activation of Erks, JNKs, and p38 kinase pathways (2, 6, 41). To study the molecular basis for neoplastic transformation activity by TNF-α, we investigated the effects on MAP kinase signal transduction pathways in our system. We found that exposure of cells to TNF-α caused the activation of MAP kinase, including Erks and p38 kinase as well as JNKs (Fig. 2), whereas TPA only induced Erks and p38 kinase (Fig. 2). The activation of JNKs and Erks by TNF-α (25 units/ml) only appears in early time points (less than 2 h), whereas p38 kinase showed sustained activation (at least 6 h) (Fig. 2 and data not shown). These data suggest that activation of MAP kinase may be involved in TNF-α-induced cell transformation.

TNF-α-induced Cell Transformation Is Blocked by Introduction of a Dominant Negative Mutant of JNKα—To determine the role of JNK activation in TNF-α-induced JB6 cell transformation, we established a dominant negative JNKα stable transfectant, Cl 41 DN JNK1 mass2. The stable transfectant was generated by “mass culture selection” of pooled clones as described previously (39). JNKs are activated by phosphorylation of Thr183 and Tyr185 by stress-activated protein kinase kinase 1 and stress-activated protein kinase kinase 4 (6), and the two other MAP kinase members, Erks and p38 kinases. We found that expression of dominant negative JNK1 did not have any blocking effects on JNK activation, we compared the JNK phosphorylation induced by UV irradiation between dominant negative transfectant Cl 41 DN JNK1 mass2 and the vector control transfectant Cl 41 CMV-neo mass1. The data show that UV-induced JNK phosphorylation was impaired by the introduction of dominant negative JNK1, whereas there were no significant effects on UV-induced Erk activation (Fig. 3). Most importantly, the cell transformation induced by TNF-α was impaired by the expression of dominant negative JNK1 (Fig. 4A). In contrast, there was no inhibition of cell transformation induced by TPA (Fig. 4A). To confirm our findings, we also established another three “mass culture” transfectants with dominant negative JNK1 mutant. The results observed from these transfectants are consistent with the above findings (Fig. 4B). These results strongly suggest that activation of JNKs may play an important role in TNF-α-induced cell transformation but not TPA-induced cell transformation.

Expression of Dominant Negative JNK1 Blocks TNF-α-induced JNK Activation—The above results indicate that TNF-α-induced cell transformation was impaired by introduction of the dominant negative JNK1 mutant; therefore, the TNF-α-induced JNK activation in this transfectant was investigated. From both time course and dose-response studies it was found that JNK activation induced by TNF-α was blocked by the expression of dominant negative JNK1 (Fig. 5). These results, taken together with the above data, demonstrate the essential role of JNKs in TNF-α-induced cell transformation.

Expression of Dominant Negative JNK1 Does Not Block TNF-α-induced Erk Activation and p38 Kinase Activation—To determine the specificity of dominant negative JNK1 in blocking signal transduction pathways, we also studied these effects on two other MAP kinase members, Erks and p38 kinases. We found that expression of dominant negative mutant JNK1 did not have any blocking effects on Erk or p38 activation, which demonstrated that JNK1 played an essential role in the TNF-α-induced cell transformation.
not show any observable inhibition of activation of Erks and p38 kinase induced by TNF-α (Fig. 6), whereas it blocked JNK activation (Fig. 5). These data demonstrate that dominant negative JNK1 blocks signal transduction specifically through the JNK pathway, suggesting that the JNK pathway is required for TNF-α-induced cell transformation, but not for TPA-induced cell transformation.

DISCUSSION

Because most of the previous studies were focused on the signal transduction pathway leading to JNK activation and their results were mainly derived from transient transfection studies, the role of JNKs in tumor promotion is still uncertain.

In the present study, we investigated this problem by establishing a stable transfectant with a dominant negative mutant JNK1. Introduction of a dominant negative mutant of JNK1 into Cl 41 cells specifically inhibited TNF-α-induced JNK activation, but not Erks and p38 kinase. Furthermore, TNF-α-induced cell transformation was impaired by the expression of dominant negative JNK1, whereas TPA-induced cell transformation was not affected. These data strongly suggest that JNK activation is at least one of the essential signals for TNF-α-induced cell transformation.

It was proposed that Erks and JNKs perform opposing functions in cell growth and apoptosis (47). However, the involvement and function of these kinases in cell proliferation, differentiation, and apoptosis have not been well defined. JNKs are more potently stimulated by inflammatory cytokines, such as TNF-α and IL-1 (24, 31), and by environmental stresses such as UV light, heat, and DNA-damaging agents (6, 20, 21, 48, 49). JNKs appear to be downstream of Rac1, cdc42, MEKK, and stress-activated protein kinase/Erk kinase (3, 28, 50). Erk activation has been linked to mitogenic stimulation in fibroblasts, but this same signaling pathway can be mediated by a completely different response in the rat pheochromocytoma PC12 cell line (51, 52). A nerve growth factor-induced Erk pathway is involved in the growth and differentiation of PC12 cells (49). It was also reported that the Erk pathway is necessary for malignant transformation by oncogenic Ha-ras (53). Activation of the Erk pathway by overexpressing constitutively active MEK mutants can also transform fibroblasts (51, 54). In addition, our studies have recently demonstrated that Erk activation plays an essential role in TPA- and EGF-induced cell transformation in a JB6 cell system (8, 55). The tumor promotion resistance in the JB6 P2 (Cl 30.7b) cells is attributable to a deficiency in basal and TPA-induced Erks (8). Moreover, the stable expression of dominant negative Erk2 in tumor promotion-sensitive cell line Cl 41 blocks the tumor promoter (TPA or EGF)-induced cell transformation (55).

In contrast, a variety of functions have also been attributed to the JNK pathway. Because both JNKs and p38 kinase are strongly activated by pro-inflammatory cytokines such as TNF-α and IL-1, it was proposed that these signaling pathways...
may have a role in inflammatory responses (56). JNKs are also thought to be involved in the induction of cytokines and chemokines as well as cyclooxygenase 2, which are considered to play a key role in the inflammatory response by catalyzing the production of prostaglandins (36). It was reported that the JNK pathway is involved in TNF-α-induced apoptosis under conditions that activate JNK in a sustained manner (57). Also, overexpression of MEKK, the JNK kinase, has a lethal effect on fibroblasts (37, 48). Overexpression of JNK1 caused cell death in the transfected cells, whereas expression of dominant negative mutants of the JNK kinase cascade blocked γ-radiation- and UVC-induced cell death (40). In this study, we found that the early activation of JNKs is required for TNF-α-induced cell transformation. The different results observed in all these groups may be caused by different cell lines, different extracellular stimuli, and the dose size used in the studies. This explanation was supported by the previous findings that in PC12 cells, JNK was proposed to trigger apoptosis in response to nerve growth factor withdrawal, whereas Erks were proposed

FIG. 4. Introduction of dominant negative JNK1 specifically blocks cell transformation induced by TNF-α but not TPA. 1 × 10⁴ of Cl 41 CMV-neo mass₁ or Cl 41 dominant negative JNK₁ stable transfectants were exposed to TNF-α (25 units/ml) or TPA (10 ng/ml) in 1 ml of 0.33% BME agar containing 15% FBS over 3.5 ml of 0.5% BME agar containing 15% FBS in each well of 6-well plates. The cultures were maintained in a 37 °C, 5% CO₂ incubator for 14 days. The cell colonies were scored after 14 days of incubation. Each column and bar indicates the mean and S.D. from triplicate assays.

FIG. 5. Blocking of TNF-α-induced JNK activation by expression of dominant negative JNK1. 8 × 10⁴ JB6 Cl 41 CMV-neo mass₁ or Cl 41 DN-JNK1 mass₂ were seeded into each well of 6-well plates. After culturing at 37 °C for 24 h, the cells were starved for 48 h by replacing medium with 0.1% FBS MEM. Four hours before cells were exposed to TNF-α, the medium was changed to serum-free MEM. Then (A) for time course study, the cells were exposed to TNF-α (25 units/ml) for the indicated time; and (B) for dose-response study, the cells were exposed to different concentrations of TNF-α for 30 min. The cells were extracted and phosphorylated, and unphosphorylated JNKs proteins were determined as described in the phosphoPlus MAPK antibody kit by New England Biolabs.

FIG. 6. Expression of dominant JNK1 mutant does not block TNF-α-induced activation of Erks or p38 kinase. JB6 Cl 41 transfectants were seeded into each well of 6-well plates. After culturing at 37 °C for 24 h, the cells were starved for 48 h by replacing medium with 0.1% FBS MEM. Four hours before cells were exposed to TNF-α, the medium was changed to serum-free MEM. The cells were exposed to 25 units/ml TNF-α for a time course study; for a dose-response study the cells were exposed to different concentrations of TNF-α for 30 min. The cells were extracted and phosphorylated, and unphosphorylated JNKs proteins were determined as described previously (41).
to inhibit apoptosis (17). However, the opposite effect was seen in B lymphocytes (58). In B cells, activation of JNKs rescues cells from apoptosis, whereas activation of Erks by activation of cell surface IgM can cause apoptosis (58). Sustained activation of JNKs by relatively high doses of TNF-α is involved in TNF-α-induced apoptosis (56). Sustained activation of UVC- and γ-radiation-induced JNKs is also responsible for apoptosis induction (40). Very recently, we demonstrated delayed activation of JNKs is required for apoptosis induced by high doses of arsenic (59).

The role of JNKs in cell proliferation and transformation has not been well studied. JNKs are strongly activated by the oncoprotein v-Src. Transient activation of JNKs has been associated with hepatic regeneration and signaling pathways leading to IL-2 induction and T cell activation (58, 60). JNK activation was also observed in v-Src and human T cell leukemia virus type I transfected cells (23). Moreover, Rodriguez et al. (22) found that dominant negative mutants of Rac block both Met-induced JNK activation and cell transformation. To establish a stable transfectant of CI 41 cells expressing a dominant negative JNKH, we have demonstrated that dominant negative JNK specifically blocks TNF-α-induced JNK activation but does not block the activation of Erks and p38 kinases. Furthermore, TNF-α-induced cell transformation in this transfectant was dramatically impaired, whereas TPA-induced cell transformation did not show any inhibition. These data provide direct evidence that activation of JNKs by TNF-α is required for its cell transformation activity. The results suggest that JNKs may be used as targets for the prevention of carcinogenesis induced by TNF-α, UV, or other JNK inducers.

Acknowledgments—We thank Dr. H. H. O. Schmid for his critical reading, Dr. Roger S. Davis for his generous gift of dominant negative JNK mutant plasmids, and Carmen Hotson and Andria Percival for secretarial assistance.

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