Implication of TNF Receptor-I-Mediated Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) Activation in Growth of AIDS-Associated Kaposi's Sarcoma Cells: A Possible Role of a Novel Death Domain Protein MADD in TNF-α-Induced ERK1/2 Activation in Kaposi's Sarcoma Cells

Kaoru Murakami-Mori, Shunsuke Mori, Benjamin Bonavida and Shuji Nakamura

*J Immunol* 1999; 162:3672-3679; http://www.jimmunol.org/content/162/6/3672
Implication of TNF Receptor-I-Mediated Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) Activation in Growth of AIDS-Associated Kaposi’s Sarcoma Cells: A Possible Role of a Novel Death Domain Protein MADD in TNF-α-Induced ERK1/2 Activation in Kaposi’s Sarcoma Cells

Kaoru Murakami-Mori, Shunsuke Mori, Benjamin Bonavida, and Shuji Nakamura

TNF-α is a key pathogenic mediator of infectious and inflammatory diseases. HIV infection stimulates and dysregulates the immune system, leading to abnormal production of TNF-α. Despite its cytotoxic effect on some tumor cell lines, TNF-α functions as a growth stimulator for Kaposi’s sarcoma (KS), a common malignancy in HIV-infected patients. However, signaling pathways linked to TNF-α-induced mitogenic responses are not well understood. We found that extracellular signal-regulated kinases 1 and 2 (ERK1/2) in KS cells were significantly activated by TNF-α through tyrosine/threonine phosphorylation. Using neutralizing anti-TNF-R-I and TNFR-II mAbs, we have now obtained evidence that TNF-α-mediated KS cell growth and ERK1/2 activation are mediated exclusively by TNFR-I, not by TNFR-II. A selective inhibitor for ERK1/2 activator kinases, PD98059, profoundly inhibited not only the activation of ERK1/2, but also the TNF-α-induced KS cell proliferation. We therefore propose that the TNF-R-I-ERK1/2 pathway plays a pivotal role in transmitting to KS cells the mitogenic signals of TNF-α. TNF-R-I possesses no intrinsic kinase activity, suggesting that TNF-R-I-associated proteins may provide a link between TNF-R-I and ERK1/2 activation.

Received for publication October 28, 1998. Accepted for publication December 9, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Boiron Research Foundation.
2 Address correspondence and reprint requests to: Dr. Kaoru Murakami-Mori, Department of Microbiology and Immunology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90095; and Institute of Molecular Medicine, Huntington Memorial Hospital, Pasadena, CA 91105.
3 Abbreviations used in this paper: KS, Kaposi’s sarcoma; Act D, actinomycin D; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain protein; MADD, mitogen-activated protein kinase-activating death domain protein; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK activator kinase; OM, oncostatin M; RIP, receptor-interacting protein; TRADD, TNF-R-I-associated death domain protein; TRAF2, TNF-R-associated factor-2; GST, glutathione S-transferase.
(26, 27). The significance of existence of two separate types of TNFRs has heretofore remained unclear. The earliest event occurring in many cells after TNF-α stimulation is rapid phosphorylation and activation of various cellular protein kinases (19). Considering a highly pleiotropic nature of TNF-α, it is conceivable that different intracellular signaling pathways play specific biological roles in TNF-α-mediated cellular responses. Mitogen-activated protein kinases, p44 MAPK and p42 MAPK (also termed extracellular signal-regulated kinases, ERK1 and ERK2, respectively), have been reported to play pivotal roles in transmitting and integrating extracellular signals required for regulation of cell proliferation, differentiation, and apoptosis (28). Despite several reports (29–32) that TNF-α can activate ERK1/2 activity, little attention has been given to its significance in TNF-α-induced proliferative events. The cytoplasmic domains of TNFRs lack intrinsic kinase activities (26, 27), suggesting that TNFR-associated proteins may be important for the activation of downstream kinase cascades. Several TNFR-associated proteins have been characterized as transducers of death signal in TNF-α-mediated cytotoxicity (33, 34); however, it has remained unclear how TNFRs transduce mitogenic signals to downstream mediator kinases.

We found that ERK1/2 in KS cells were significantly activated by TNF-α. Using neutralizing TNFR-I- and TNFR-II-specific mAbs, we determined which type of TNFRs mediates TNF-α-induced KS cell growth and ERK1/2 activation. To evaluate the commitment of ERK1/2 signaling pathway in the mitogenic action of TNF-α for KS cells, we used PD98059, a selective inhibitor of the ERK1/2 activator kinases. We propose that TNF-α elicits the mitogenic responses of KS cells through the TNFR-I-ERK1/2 signaling pathway. In addition, we searched for a possible role of MADD, a novel TNFR-I-associated protein (35), to bridge a molecular gap between the activation of TNF-α and the triggering of mitogenic response of KS cells through the TNFR-I-ERK1/2 signaling pathway.

Materials and Methods

Cells and reagents

AIDS-KS cells were developed from pleural effusion of lung KS (KS22 and KS23), lung KS (KS3), oral mucosa KS (KS10B), and skin KS (KS14), all from different HIV-1-infected patients. KS22 and KS23 cells were established in the Institute of Molecular Medicine, Huntington Memorial Hospital (Pasadena, CA) (36), and KS3, KS14, and KS10B cells were developed in the Laboratory of Tumor Cell Biology, National Cancer Institute, Institute of National Health (Bethesda, MD) (13). These KS cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD), supplemented with 10% FBS (Gemini Bio-Products, Calabasas, CA) and conditioned medium from human oncostatin M (OM) expressing Chinese hamster ovary cells, to a final OM concentration of 10 ng/ml. OM-expressing Chinese hamster ovary cells were established earlier (36). Human recombinant TNF-α, IL-1β, and OM, and neutralizing mouse anti-human soluble TNFR-I and TNFR-II mAbs were purchased from R&D Systems (Minneapolis, MN). Neutralizing mouse anti-human TNF-α mAb and mouse control IgG were also purchased by R&D Systems. PD98059 and actomyosin D (Act D) were purchased from Calbiochem-Novabiochem (La Jolla, CA) and Sigma (St. Louis, MO), respectively.

AIDS-KS cell growth assays

AIDS-KS cells were incubated in triplicate in 24-well plates (0.5 ml/well) in KS basal medium (RPMI 1640, 10% FBS), with or without various test factors, at an initial cell density of 3 × 10^4 cells/well. To determine the effect of PD98059, KS cells were preincubated with this reagent for 30 min before addition of TNF-α. Culture medium was changed every 3 days, and cells were counted on the sixth day of culture, using a Coulter particle counter (Coulter Electronics, Hialeah, FL). Data are expressed as the mean ± SD of triplicate determinations from three separate experiments. The criterion that cell death should be less than 1% was met by all growth assays included in this study, even in the presence of PD 98059.

RNA preparation and PCR amplification

Total RNA was prepared by guanidine isothiocyanate disruption of cells and centrifugation through 1.5 ml cesium trifuoroacetate (CSF/TA; Pharmacia, Piscataway, NJ). Two micrograms of total RNA prepared from four AIDS-KS cells isolates were subjected to cDNA synthesis by incubation for 1 h at 42°C with reverse transcriptase and random hexanucleotides (Life Technologies), followed by TNFR-I-, TNFR-II-, and MADD-specific PCR amplification, respectively. The primers used were: 5′-TACCAACGGTGGAAGTCCAAGCT-3′ and 5′-GATCTCGAGGAGTGCTGATG-3′ (for TNFR-I), 5′-CGGGCACTGTCAGATGATG-3′ and 5′-TTAATCTGGCTTCTACCATCGCC-3′ (for TNFR-II); and 5′-AAGATGAGCGCCCTAATTGGG-3′ and 5′-CATGACCTAGACACGGCAG-3′ (for MADD). RNA integrity and the efficiency of cDNA synthesis were confirmed by PCR amplification, using a human β-actin-specific primer (Ciontech, Palo Alto, CA). PCR amplification was done under the following condition: 35 cycles at 94°C for 1 min, at 58°C for 2 min, and at 72°C for 3 min; incubation at 94°C for 1 min, at 58°C for 2 min, and at 72°C for 3 min. The amplification products were electrophoresed on 1.5% agarose gels.

Cytokine stimulation of AIDS-KS cells for in vitro kinase assays and Western blotting analysis

ERK1/2 is weakly phosphorylated in AIDS-KS cells grown in the KS basal medium. To reduce this basal level of phosphorylation, KS cells (2×10^6 cells) in a 75-cm² culture flask were deprived of serum for 3 days in serum-free medium (RPMI 1640, 0.4% BSA). These cells were then pre-treated for 10 min with 1 mM NaF and stimulated with either 10 ng/ml of TNF-α, IL-1β, OM, or OM for various periods at 37°C. To examine effects of blocking of TNFR-I- or TNFR-II-mediated signals, KS cells were pre-treated with 10 μg/ml neutralizing anti-human soluble TNFR-I or TNFR-II mAb for 1 h before TNF-α stimulation. To inhibit the ERK1/2 activity, KS cells were preincubated with the indicated concentrations of PD98059 for 30 min before TNF-α stimulation. To examine effects of Act D, KS cells were treated with Act D (10 ng/ml) for 1 h before cytokine stimulation. These cells were lysed at 4°C in 1 ml of lysis buffer for kinase assays (50 mM HEPES (pH 7), 0.1% Nonidet P-40, 250 mM NaCl, 2 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, and 1 mM NaF).

In vitro kinase assays

The cell lysates (200 μg of proteins in 200 μl) were preincubated by incubation for 1 h at 4°C with 1 μg of rabbit IgG together with 20 μl of 50% suspension of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The lysates were incubated overnight at 4°C with rabbit anti-human ERK1/2 polyclonal Ab (1 μg; Santa Cruz Biotechnology). The immune complexes were then isolated by incubation with 20 μl of 50% suspension of protein A/G agarose for an additional 1 h at 4°C. After washing four times with the mouse IgG buffer, ELK-1 was eluted with 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 1 mM Na3 VO4, and 1 mM NaF), the immunoprecipitates were incubated for 30 min at 30°C in 25 μl of the kinase buffer, in the presence of 2 μg of GST-Elk-1 fusion protein containing the carboxyl-terminal domain of Elk-1 (New England Biolabs, Beverly, MA) as a substrate for ERK1/2, 3 μCi of [γ-32P]ATP (6000 Ci/mmol; Amersham, Arlington Heights, IL) was electrophoretically conducted in a transblotting cell (Bio-Rad, Hercules, CA). Membranes were blocked by immersing for 1 h at room temperature in 5% nonfat skim milk/PBS, then the membranes were incubated with rabbit anti-ERK1/2 or anti-phospho-specific ERK1/2 polyclonal Ab (2 μg/ml; New England Biolabs), goat anti-TRADD (0.5 μg/ml; Santa Cruz Biotechnology), rabbit anti-MEK1/2 polyclonal Ab (0.5 μg/ml; Santa Cruz Biotechnology), mouse anti-FADD or anti-RIP mAb (1 μg/ml; Transduction Laboratories, Lexington, KY), or rabbit anti-TRAF2 polyclonal Ab (0.5 μg/ml; Medical Biological Laboratories, Nagoya, Japan) for 2 h at room temperature. The anti-phospho-specific ERK1/2 Ab detects ERK1/2 only when activated by phosphorylation at threonine 202 and tyrosine 204 residues, and the anti-ERK 1/2 Ab detects total ERK1/2 proteins (phosphorylation state-independent levels). After washing in PBS/0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary Ab (New England Biolabs), followed by NBT/BCIP (Promega, Madison, WI) for 1 h at room temperature. Finally, membranes were stained with QuickBlue (Bethesda Research Laboratories, Gaithersburg, MD).
or anti-goat IgG secondary Ab (Sigma), and then developed using a Phototope-horseradish peroxidase Western blot detection kit (New England Biolabs).

**Results**

**Growth-stimulating effects of TNF-α on AIDS-KS cells**

To confirm the growth-stimulating effect of TNF-α on KS cells, we conducted growth assays on three different isolates of KS cells (KS3, KS10B, and KS22), in the presence or the absence of increased concentrations of TNF-α (Fig. 1). In the absence of TNF-α, KS cells showed only minimum levels of growth on the sixth day of culture. When increased concentrations of TNF-α were added to the KS cell cultures, these cells proliferated in a dose-dependent manner. Maximum growth levels, which were obtained with 5 ng/ml of TNF-α, were 3- to 3.5-fold higher than the basal growth levels observed with medium alone. Thus, TNF-α functions as a mitogen for KS cells.

**Involvement of TNFR-I in TNF-α-induced proliferation of AIDS-KS cells**

The biological effects of TNF-α are transmitted via two distinct membrane receptors, TNFR-I and TNFR-II (26, 27). Using total RNA prepared from four isolates of KS cells (KS3, KS10B, KS14, and KS22), the expression of specific mRNA for TNFR-I and TNFR-II was demonstrated by the appearance of RT-PCR amplification products. As shown in Fig. 2A, considerable amounts of PCR products specific for TNFR-I and TNFR-II were observed with all three KS isolates.

In attempts to determine which type of TNFRs is responsible for TNF-α-induced stimulation of KS cell growth, increased amounts of neutralizing mAbs specific for TNFR-I and TNFR-II were added to KS cell cultures (KS3, KS10B, and KS22), in the presence of 5 ng/ml of TNF-α. As shown in Fig. 2B, the anti-TNFR-I mAb profoundly inhibited the TNF-α-induced growth, in a dose-dependent fashion, and complete inhibition was obtained with 10 μg/ml of anti-TNFR-I mAb. In contrast, effects of anti-TNFR-II mAb were hardly detectable. Thus, the mitogenic effects of TNF-α on KS cells apparently occur through TNFR-I-mediated signaling events, while the role of TNFR-II on KS cell proliferation, if any, seems minimal.

**Tyrosine/threonine phosphorylation and activation of ERK1/2 by TNF-α treatment of AIDS-KS cells**

It has been reported that TNF-α can induce activation of ERK1/2 (29–32). This activation apparently occurs through dual phosphorylation of tyrosine and threonine residues (37). To determine the activation state of ERK1/2 in TNF-α-treated KS cells, we examined the tyrosine/threonine phosphorylation of ERK1/2 and its kinase activity. Following serum deprivation for 3 days, these cells...
were incubated with 50 ng/ml of TNF-α for the indicated periods, then subjected to immunoprecipitation for ERK1/2. In vitro kinase assays for ERK1/2 were done using its physiologic substrate Elk-1 (38). To detect tyrosine/threonine-phosphorylated ERK1/2 proteins, Western blotting of these immune complexes was done using an anti-phospho-ERK1/2 Ab. As shown in Fig. 3, only little phosphorylated bands of ERK1/2 were hardly detected in these cells. High levels of kinase activities of immunoprecipitated ERK1/2 were observed in quiescent KS cells. Tyrosine/threonine-phosphorylated bands of ERK1/2 were hardly detected in these cells. High levels of kinase activities were induced within 10 min after TNF-α stimulation and continued for at least 120 min. In parallel, TNF-α stimulation induced a large accumulation of tyrosine/threonine-phosphorylated ERK1/2 in the KS cells. Using an anti-ERK1/2 Ab, we confirmed that similar levels of ERK1/2 proteins were seen in all lanes of the time course tested. Taken together, it seems evident that TNF-α has the potential to activate ERK1/2 in KS cells.

**FIGURE 3.** Tyrosine/threonine phosphorylation of ERK1/2 and its activation in TNF-α-treated AIDS-KS cells. KS cells were deprived of serum for 3 days and then stimulated with 50 ng/ml of TNF-α for 0, 10, 30, 60, and 120 min at 37°C. In vitro kinase assays (upper panel): cell lysates (200 μg) prepared from these cells were subjected to immunoprecipitation with anti-ERK1/2 Ab. The immunoprecipitates were incubated for 30 min at 30°C in 25 μl of the kinase buffer containing 5 μCi of [γ-32P]ATP (6000 Ci/mmol) and 50 μM cold ATP, in the presence of 1 μg of GST-Elk-1 as a substrate. The samples were separated by 12% SDS-PAGE and visualized by autoradiography. Western blotting: cell lysates (20 μg) were subjected to 12% SDS-PAGE and immunoblotting with anti-phospho-ERK1/2 Ab (middle panel) and anti-ERK1/2 Ab (lower panel), respectively. Representative data are from four separate experiments using KS3, KS10B, and KS22 cells. Addition of 0.1% DMSO used as a solvent had little effect on ERK1/2 activation (data not shown).

**Inhibitory effects of the neutralizing anti-TNF-RI mAb on tyrosine/threonine phosphorylation and activation of ERK1/2 in AIDS-KS cells**

To determine which type of TNFRs mediates ERK1/2 activation in KS cells, we examined the effects of neutralizing anti-TNF-RI- and anti-TNF-RII-specific mAbs on the TNF-α-induced activation of ERK1/2 in KS cells, using in vitro kinase assays and tyrosine/threonine phosphorylation assays (Fig. 4). By serum starvation for 3 days before TNF-α stimulation, ERK1/2 was maintained in a quiescent state. When KS cells were stimulated with 50 ng/ml of TNF-α for 30 min, ERK1/2 activities were drastically induced, and large amounts of tyrosine/threonine-phosphorylated ERK1/2 proteins were detected on immunoblots. This activation was not observed in anti-TNF-RI mAb-treated KS cells, thereby indicating that the TNF-α-induced activation of ERK1/2 is initiated by TNF-RI. Blocking the TNF-RII pathway by a specific Ab had little or no effects on the tyrosine/threonine phosphorylation and kinase activity of ERK1/2.

**Blockage of TNF-α-induced proliferation of AIDS-KS cells by PD98059, a selective inhibitor of the MEK1/2-ERK1/2 pathway**

Several lines of evidence show that the ERK1/2 activator kinases are MEK1/2 that locate upstream in the ERK1/2 signaling pathway (39, 40). To obtain direct evidence that the MEK1/2-ERK1/2 pathway has a crucial role in KS cell growth, a selective inhibitor of MEK1/2, PD98059 (41), was used in the growth assays (Fig. 5A). Treatment with TNF-α (10 ng/ml) generated high levels of KS cell growth. When KS cells were preincubated with PD98059 for 30 min before TNF-α stimulation, this inhibitor abrogated the TNF-α-induced proliferation, in a dose-dependent manner, with an apparent IC₅₀ of about 10 μM. Trypan blue dye exclusion showed that cell viability in all growth assays exceeded 95%. The growth-inhibitory activity of PD98059 was reversible, because removal of this inhibitor led to a resumed proliferation in response to TNF-α (data not shown). To confirm that the pretreatment with PD98059 blocks the activation of the MEK1/2-ERK1/2 pathway in KS cells, the phosphorylation state of ERK1/2 was determined using Western blotting for tyrosine/threonine-phosphorylated ERK1/2 (Fig. 5B). Following stimulation with TNF-α for 30 min, significant levels of tyrosine/threonine-phosphorylated ERK1/2 accumulated in KS cells. Pretreatment of the KS cells with PD98059 dose dependently suppressed this phosphorylation. Thus, cellular activity of the MEK1/2-ERK1/2 pathway is required for the TNF-α-induced growth of KS cells.

**Selective inhibition of MADD expression in Act D-treated AIDS-KS cells**

As shown above, the mitogenic activity of TNF-α is mediated through the TNFR-I-MEK1/2-ERK1/2 signaling pathway in KS...
Since the intracellular regions of TNFR-I lack intrinsic kinase activities (26, 27), TNFR-I-associated proteins may be important for activation of the downstream MEK1/2-ERK1/2 cascade. Schievella et al. isolated a protein called MADD, which associates with a cytoplasmic region of TNFR-I termed death domain; they reported that overexpression of this protein in COS cells induced activation of ERK2 proteins (35). RT-PCR amplification, using total RNA of four different isolates of KS cells (KS3, KS10B, KS22, and KS23), revealed considerable amounts of expression of MADD mRNA (Fig. 6A). In addition, we noted that treatment with 10 ng/ml of Act D led to a complete inhibition of MADD expression in all isolates of KS cells tested. In contrast, Act D treatment did not affect expressions of TNFR-I and TNFR-II. TRADD, a member of the death domain protein family, also interacts directly with TNFR-I through the death domain (42). Western blotting of cell lysates prepared from KS3 and KS10B showed that the protein levels of TRADD were not affected by Act D treatment (Fig. 6B).

TRADD is believed to act as an adapter molecule that recruits three signal-transducing molecules, TRAF2, RIP, and FADD, to TNFR-I, thereby resulting in formation of TNFR-I signaling complexes (33, 34, 43, 44). As shown in Fig. 6B, Act D had no effect on expression of these proteins in KS cells. In addition, expressions of MEK1/2 and ERK1/2 proteins were not affected by Act D treatment of KS cells. This concentration of Act D had no cytotoxic effects on KS cells used in this study (45).
MADD provides a link between TNFR-I and ERK1/2 activation in KS cells, thereby leading to proliferative responses of these cells.

**Discussion**

The majority of biological responses attributed to TNF-α seems to be mediated by TNFR-I; however, functional roles of TNFR-I and TNFR-II in the control of cell growth may depend on the cell type (26, 27). For instance, proliferative responses of fibroblasts are controlled by TNFR-I, while growth stimulation of thymocytes and cytotoxic T cells is attributed to TNFR-II clustering (46–48). Both receptors are required for proliferation of NK cells, PBMCs, and megakaryoblastic leukemic cells (49–51). In our present study, we found that the neutralizing mAb directed against TNFR-I completely inhibits TNF-α-induced KS cell proliferation, whereas neutralizing TNFR-II-specific mAb had little or no effect on growth (Fig. 2B). This means that proliferative signals of TNF-α for KS cells are exclusively mediated by activating TNFR-I. Such selective inhibition of growth in the presence of neutralizing anti-TNF-α mAb cannot be explained simply by differential expression of these receptors, since we show that KS cells coexpress both types of TNFRs (Fig. 2A). It has been postulated that there is a unique activity associated with TNFR-II; that is, TNF-α can greatly enhance TNFR-I-mediated cellular responses by recruiting TNF-α to the cell surface and delivering it to neighboring TNFR-I (52). Since TNFR-II has a higher affinity ($K_d$ value of 0.1 nM) than TNFR-I ($K_d$ value of 0.5 nM), TNF-α may first bind to TNFR-II, at low ligand concentrations (26). However, this event seems unlikely for the TNF-α-induced proliferation of KS cells, since inhibitory effects of neutralizing anti-TNF-II mAb on KS cell growth were not observed even at the lowest concentration of TNF-α required for growth induction (data not shown).

TNF-α significantly activated the ERK1/2 signaling pathway in KS cells (Fig. 3). Using the MEK1/2-specific inhibitor PD98059, we obtained evidence that the TNF-α-induced ERK1/2 activation is required to trigger proliferative responses of KS cells (Fig. 5). Amaral et al. reported that OM stimulated tyrosine phosphorylation and activation of ERK2, and inhibitors of protein tyrosine phosphorylation profoundly suppressed OM-induced KS cell growth (53). We showed that OM and IL-1β can induce ERK1/2 activation in KS cells (Fig. 7B). In addition, PD98059 completely inhibited OM- and IL-1β-induced growth of KS cells (K. Murakami-Mori, unpublished observations). Taken together, it seems apparent that the activation of ERK1/2 is an absolute requirement for proliferative response of KS cells. On the other hand, we reported that the simple treatment with acidic or basic fibroblast growth factor alone is inadequate for growth promotion of KS cells, although fibroblast growth factors are known as strong activators of ERK1/2 (25). Activation of an additional signaling pathway(s) may be required for KS cell proliferation. Little is known about the intracellular signaling pathway by which TNF-α exerts its mitogenic activity. Dionne et al. reported that TNF-α-induced activation of ERK1/2 is required for growth of rat intestinal crypt cells (54). In contrast, TNF-α promoted growth of human megakaryoblastic leukemia cells, but this cytokine failed to activate ERK1/2 in these cells, thereby suggesting that the ERK1/2 signaling pathway is not involved in TNF-α-induced proliferation (51). TNF-α is also known to activate other MAPK family members, JNK and p38 MAPK (19). We noted weak activation of JNK and p38 MAPK in TNF-α-treated KS cells (data not shown). Involvement of these MAPKs in TNF-α-induced proliferation of KS cells is currently under investigation.

We consider that TNFR-I mediates the TNF-α-induced phosphorylation of ERK1/2 and its activation in KS cells, as based on
the potential of antagonistic anti-TNF-α mAb to inhibit these responses (Fig. 4). In contrast, TNF-α does not participate in the ERK1/2 signaling pathway in KS cells, since the TNFR-α-specific neutralizing mAb had little effect on phosphorylation and activation of ERK1/2 in these cells (Fig. 4). Considering that the intracellular regions of TNFR-α and TNFR-β are structurally unrelated (26, 27), it is conceivable that these two receptors are responsible for induction of distinct intracellular signaling events. In spite of the ability of TNF-α to phosphorylate and activate ERK1/2, the cytoplasmic domains of TNFR-I have no intrinsic kinase activity (26, 27). The cytoplasmic death domain of TNFR-I is known to mediate a direct interaction of this receptor with the adapter molecule TRADD, which recruits the signal-transducing molecules FADD, TRAF2, and RIP. Such a TNFR-I-associated signaling complex has been implicated in the initiation of an apoptotic response and the activation of nuclear factor-κB (33, 34, 42–44). TRAF2 and RIP also mediate the JNK activation; however, none of these signaling molecules is able to induce ERK1/2 activation, as evidenced in experiments using overexpression of these proteins (55). In the present study, we show the possibility that the novel TNFR-I-associated death domain protein, MADD, is associated with TNFR-α-mediated activation of ERK1/2 in KS cells: TNF-α failed to induce ERK1/2 activation in Act D-treated KS cells, in which MADD expression was completely and selectively suppressed (Figs. 6 and 7). Expressions of other components of the TNFR-I signaling complex were not influenced by Act D treatment of KS cells (Fig. 6). Schievela et al. showed that MADD interacts with TNFR-I and TRADD through their death domains. Overexpression of this protein had no effect on the induction of apoptosis, but it induced robust activation of ERK1/2 in COS cells (35). Taken together, it seems reasonable to assume that MADD links TNFR-I to the ERK1/2 activation for triggering the proliferative response of KS cells. Small GTP-binding proteins (G proteins), such as Ras, CDC42, and Rac, have been implicated in activation of ERK1/2 and JNK signaling cascades (56, 57). Most recently, Brown et al. showed that MADD is highly homologous to a member of the GDP/GTP exchange protein family that plays an important role in generation of GTP-bound active forms of G proteins (58). These findings may suggest that MADD participates in the TNFR-I-ERK1/2 signaling pathway via activation of G proteins. Further studies on the molecular basis of interactions between MADD and G proteins in KS cells may provide new insights into the mechanism by which TNF-α elicits the mitogenic response of KS cells.

TNF-α has been considered a target for KS therapy, since this cytokine is known to play a pivotal role in inflammatory responses and angiogenic changes, characteristic features of KS lesions (59). In addition, high levels of TNF-α expression are evident in KS lesions (23). Aggravated development of KS lesions has been noted during times of opportunistic infections, events probably associated with increased serum TNF-α levels in AIDS patients (7). Thus, TNF-α may provide conditions favorable for KS progression. Epidemiologic studies show that KS is associated with a novel human herpesvirus-8 infection (60). Since this virus productively infects mononuclear cells infiltrating KS lesions, this virus may contribute to development and maintenance of KS lesions by triggering local inflammatory cytokine production (61, 62). Because KS cell is a regulatory element in this lesion, mitogenic cytokines for KS cell could be good targets for blocking KS development and progression, at least during the early stages of the disease (10). Our evidence shows that TNF-α induces KS cell proliferation through direct activation of the TNFR-I-ERK1/2 pathway, and that treatment of KS cells with anti-TNF-α mAb or PD98059 profoundly inhibits proliferation of these cells. Such knowledge should lead to a therapeutic approach for anti-KS treatment. Development of pathogenesis-based therapies that provide tumor specificity can be considered.

Acknowledgments

We thank M. Ohara for critical readings of the manuscript.

References


15. Murakami-Mori, K., T. Taga, T. Kishimoto, and S. Nakamura. 1996. The soluble form of the IL-6 receptor (sIL-6Rα) is a potent growth factor for AIDS-associated Kaposi’s sarcoma (KS) cells: the soluble form of gp130 is antagonistic for sIL-6Rα-induced AIDS-KS cell growth. Int. Immunol. 8:595.


