Apoptosis-inducing Agents Cause Rapid Shedding of Tumor Necrosis Factor Receptor 1 (TNFR1)

A nonpharmacological explanation for inhibition of TNF-mediated activation

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Several chemical compounds not known to interact with tumor necrosis factor (TNF) signal transducing proteins inhibit TNF-mediated activation of vascular endothelial cells (EC). Four structurally diverse agents, arachidonyl trifluoromethylketone, staurosporine, sodium salicylate, and C6-ceramide, were studied. All four agents caused EC apoptosis at concentrations that inhibited TNF-induced IxB degradation. However, evidence of apoptosis was not evident until after several (e.g. 3–12) hours of treatment, whereas 2 h of treatment was sufficient to inhibit TNF responses. IL-1-induced IxBa degradation was unaffected by these treatments. Inhibition of TNF signaling could not be prevented with either of the broad spectrum caspase inhibitors zVAD-fmk or yVAD-cmk. The inhibition of p38 kinase with SB203580 prevented the inhibition of TNF signaling by all agents except arachidonyl trifluoromethylketone. No changes in the levels or molecular weights of the adaptor proteins TRADD (TNF receptor-associated death domain), RIP (receptor-interacting protein), or TRAF2 (TNF receptor-associated factor-2) were caused by apoptogenic drugs. However, TNF receptor 1 (TNFR1) surface expression was significantly reduced by all four agents. Furthermore, TNF-dependent recruitment of TRADD to surface TNFR1 was also inhibited. These data suggest that several putative inhibitors of TNF signaling work by triggering apoptosis and that an early event coincident with the initiation of apoptosis, preceding evidence of injury, is loss of TNFRI. Consistent with this hypothesis, cotreatment of EC with the metalloproteinase inhibitor Tapi (TNF-α proteinase inhibitor) blocked the reduction in surface TNFR1 by apoptogenic drugs and prevented inhibition of TNF-induced IxB degradation without blocking apoptosis. TNFR1 loss could be a mechanism to limit inflammation in response to apoptotic cell death.

The biological effects elicited by cytokines such as TNF1 and IL-1, which include inflammation and tissue injury, are initiated by ligand-induced formation of distinct multireceptor complexes. Activation of the TNF signal transduction cascade is initiated by the interaction of TNF with two distinct surface receptors, TNFR1 (55 kDa) and TNFR2 (75 kDa) (reviewed in Ref. 1). Although both TNFR1 and TNFR2 may mediate the activation of independent downstream signaling pathways (2–4), there is evidence that TNFR2 functions primarily to bind ligand rapidly, passing soluble TNF trimers to TNFR1 (5, 6). Ligand-induced clustering of TNFR1 leads to the recruitment of a cytosolic adaptors protein, called TNF receptor 1-associated death domain protein (TRADD), to the intracellular domain of the receptor (7). Receptor-associated TRADD further recruits a protein first described as Fas-associated death domain protein, thought to lie upstream of the caspase cascade and initiation of apoptosis (8). TRADD also mediates the binding of the protein kinase RIP (receptor-interacting protein) and the ring/zinc finger protein, TNF receptor-associated factor-2 (TRAF2) (8, 9). Recent evidence from studies of rip−/− and traf2−/− knockout mice suggests that RIP is essential for the activation of the transcription factor NFκB, whereas TRAF2 is required for activation of the transcription factor AP-1 (10–12). More specifically, RIP may activate the TRAF2-interacting NFκB-inducing kinase (13, 14), which activates two recently identified kinases, IκB kinase-1 and -2, that exist in a multikinase cytosolic complex (15–18). IκBα is normally located in the cytosol of unstimulated cells in a complex with NFκB inhibiting the transcriptional activity of NFκB by masking its nuclear localization sequence (19). Upon phosphorylation by IκB kinase-1 and -2 (15–18), IκBα is rapidly ubiquitinated and degraded by the proteosome, thereby releasing NFκB, which is now free to translocate to the nucleus and induce transcription (20, 21).

IL-1, like TNF, activates NFκB and AP-1 but generally does not initiate apoptosis. IL-1-stimulated activation of NFκB is also dependent on the formation of a ligand-induced receptor complex. The occupied type 1 IL-1 receptor interacts with a transmembrane protein termed IL-1 receptor accessory protein (22) and a cytosolic adaptors molecule, MyD88 (23). This complex then binds IL-1 receptor-associated kinase (23, 24). Within the assembled type 1 IL-1 receptor complex, IL-1 receptor-associated kinase becomes autophosphorylated (25) and subsequently leaves the complex to associate with cytosolic TRAF6 (26). The IL-1 receptor-associated kinase/TRAF6 complex, like the TRADD-RIP–TRAF2 complex, lies upstream of the activation of NFκB-induced kinase, creating a point of convergence with the TNF signaling pathway.
Materials—Recombinant human TNF was a gift from Biogen (Cambridge, MA). Recombinant human IL-1β was purchased from R & D Systems Inc. (Minneapolis, MN). zVADfmk was purchased from Enzyme Systems Products (Dublin, CA). SB203580, C6-ceramide, and staurosporine (stauro) have been reported to inhibit TNF-mediated activation of NFκB, expression of E-selectin, or both in EC (29–32). However, none of these agents has been shown to interact with any of the molecules identified to date in the TNF signaling pathway. During the course of our investigation of the signaling role of arachidonic acid, we noted that concentrations of this lipid or its structural analogues, such as the cytosolic phospholipase A2 inhibitors arachidonil trifluoromethylketone (ATK) and methylarachidonil fluorophosphonate, inhibited E-selectin induction but only at concentrations that induced apoptosis. However, these agents were able to block the activation of NFκB before morphological evidence of apoptosis became detectable. These observations led us to hypothesize that coincident with the induction of apoptosis is a common mechanism by which diverse pharmacological compounds can selectively inhibit the EC response to TNF. Here we present evidence in support of this hypothesis and identify the loss of TNFR1 surface expression as the mechanism by which apoptogenic drugs selectively inhibit TNF responses.

EXPERIMENTAL PROCEDURES

Cell Culture—In accordance with an approved protocol by the Yale University Human Investigations Committee, human umbilical vein EC were isolated and cultured as described previously on gelatin (J. T. Baker Inc.-coated tissue culture plastic (Falcon, Lincoln Park, NJ) in medium 199 (M199) supplemented with 20% fetal calf serum, 200 μM l-glutamine, 100 μg/ml streptomycin, and 50 μg/ml penicillin. The cytokine-dependent transcription of this adhesion molecule that mediates the initial tethering and rolling of neutrophils. Vascular endothelial cells (EC) are a major target for the pro-inflammatory actions of TNF and IL-1. One of the major proinflammatory responses in EC initiated by the TNF and IL-1 signal transduction cascades described above is the expression of the leukocyte adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). E-selectin is a surface glycoprotein that mediates the initial tethering and rolling of neutrophils. The cytokine-dependent transcription of this adhesion molecule has an absolute requirement for the activation of NFκB (27) and can be significantly enhanced by the co-binding of ATF2/c-Jun heterodimers (a form of AP-1) to the E-selectin enhancosome (28). Inhibition of the activation of NFκB or AP-1 in response to TNF or IL-1 is therefore a potential target for novel anti-inflammatory reagents. A wide variety of diverse pharmacological agents including sodium salicylate, arachidonic acid, short chain ceramide (C₄-ceramide), and staurosporine (stauro) have been reported to inhibit TNF-mediated activation of NFκB, expression of E-selectin, or both in EC (29–32). However, none of these agents has been shown to interact with any of the molecules identified to date in the TNF signaling pathway. During the course of our investigation of the signaling role of arachidonic acid, we noted that concentrations of this lipid or its structural analogues, such as the cytosolic phospholipase A2 inhibitors arachidonil trifluoromethylketone (ATK) and methylarachidonil fluorophosphonate, inhibited E-selectin induction but only at concentrations that induced apoptosis. However, these agents were able to block the activation of NFκB before morphological evidence of apoptosis became detectable. These observations led us to hypothesize that coincident with the induction of apoptosis is a common mechanism by which diverse pharmacological compounds can selectively inhibit the EC response to TNF. Here we present evidence in support of this hypothesis and identify the loss of TNFR1 surface expression as the mechanism by which apoptogenic drugs selectively inhibit TNF responses.

RESULTS

Diverse Pharmacological Agents That Induce EC Apoptosis Block TNF but Not IL-1 Signaling—Reports from the literature and our own observations indicate that the cytosolic phospholipase A2 inhibitor ATK, the kinase inhibitor stauro, the anti-inflammatory agent sodium salicylate, and the anti-permeant

4,6-Diamidino-2-phenylindole•HCl Staining of EC—For experimental manipulation, EC were plated on human plasma fibronectin-coated multiwell chamber slides (Falcon). After treatment, cells were fixed by the addition of an equal volume of paraformaldehyde (1% final) in PBS for 30 min at room temperature. The slide was then washed twice in PBS, permeabilized for 30 s in PBS containing 0.1% Triton X-100, washed with 0.1% gelatin in PBS, and covered with a further gelatin-anti-TNFα coating. After experimental manipulation, medium was removed, and cells were washed twice in PBS. The remaining cells were fixed and stained by the addition of 70% ethanol containing 100 μg/ml Hoescht 33258 reagent (Molecular Probes) for 30 min at room temperature. Cells were again washed twice with PBS before fluorescence was recorded (λₑₒ = 360 nm, λₘᵢₐₓ = 460 nm) using a fluorescence plate reader (Perspective Biosystems Inc., Framingham, MA).

Indirect Immunofluorescence and FACs Analysis—Indirect immunofluorescence was used to quantify the surface amount of TNFR1 and TNFR2 on cultured EC. EC seeded on three 10-cm dishes (approximately 10⁶ cells/sample) in a total volume of 3 ml of M199. The lysis were centrifuged at 735 g for 10 min at 4 °C before precleared by incubation with 25 μl of a 1:1 slurry of Gamma Bind Sepharose (Amersham Pharmacia Biotech) for 2–3 h at 4 °C on a rocking platform. After centrifugation at 14,000 rpm for 10 s, the cleared lysates were transferred to another tube and incubated with 1μl/sample of TNFR1 or TNFR2 mAb to poly(A/D/E/F)-ribonuclease (PARP) was a gift from G. Poirier, CHUL Research Institute for the Expression of the following proteins: (a) ATF2, which activates NFκB, expression of E-selectin, and our own observations indicate that the cytosolic phospholipase A2 inhibitor ATK, the kinase inhibitor stauro, the anti-inflammatory agent sodium salicylate, and the anti-permeant
ceramide analogue, C<sub>e</sub>-ceramide, are all able to inhibit TNF-induced adhesion molecule expression (29, 31, 32). This inhibition of E-selectin expression is paralleled by an inhibition of TNF-induced degradation of inhibitory protein I<sub>K</sub>B<sub>B</sub>. Optimal inhibitory concentrations, determined in preliminary experiments, are 50 µM ATK, 100 nM stauro, 20 mM sodium salicylate, and 50 µM C<sub>e</sub>-ceramide. We have found that these concentrations of all of these agents cause EC to undergo apoptosis; suboptimal inhibitory concentrations were less apoptogenic (Fig. 1 and data not shown). This correlation between inhibition of TNF responses and induction of apoptosis may have been missed in prior studies (29, 31, 32) because biochemical and morphological evidence of apoptosis, such as PARP cleavage or nuclear condensation, typically did not become apparent for several (3–12) hours, the precise timing depending on the drug used (data not shown). More importantly, no evidence of apoptosis was ever detectable in the first 2 h of treatment, by which time inhibition of TNF-mediated activation was already evident.

Cytokine-induced E-selectin expression is absolutely dependent on the activation of NFκB (27). Therefore we examined the effect of 2 h of pretreatment of each apoptosis-inducing reagent on TNF- and IL-1-dependent degradation of I<sub>K</sub>B<sub>B</sub>, a critical step in the NFκB pathway. As shown in Fig. 2, A and B, TNF-induced degradation of I<sub>K</sub>B<sub>B</sub> is significantly reduced following 2 h of pretreatment with each apoptogenic drug. Surprisingly, no inhibition of I<sub>K</sub>B<sub>B</sub> degradation induced by IL-1 is detectable in replicate cultures. The observed effect of apoptogenic agents on TNF-dependent degradation of I<sub>K</sub>B<sub>B</sub> is not simply a delay in the kinetics of the degradation of this protein, because a complete time course of TNF treatment from 5 to 60 min revealed that the degradation of I<sub>K</sub>B<sub>B</sub> is inhibited at all times examined (data not shown). These observations led us to hypothesize that an early common event in the biochemical program of apoptosis selectively inhibits TNF (but not IL-1) signal transduction.

**Inhibition of TNF-induced I<sub>K</sub>B<sub>B</sub> Degradation in EC Is Not Dependent on the Activation of Caspases or p38 Kinase**—We next examined whether specific effector molecules implicated in the death pathway could inhibit TNF signal transduction. Activation of effector caspases is a common late step in apoptosis (35). To determine whether the activation of caspases participated in the inhibition of TNF-mediated NFκB activation by pretreatment with inducers of apoptosis, we employed the broad spectrum peptide-based caspase inhibitor zVADfmk. This caspase inhibitor (40 µM) was unable to prevent inhibition of TNF-mediated I<sub>K</sub>B<sub>B</sub> degradation when administered during pretreatment with ATK (50 µM) over a 2-h period (Fig. 3A) even though it was able to inhibit the characteristic caspase-dependen
Inhibition of TNF Signaling by Initiation of Apoptosis

The differential effects of apoptosis inducers on TNF- and IL-1-dependent degradation of IκBα suggest that the point of inhibition caused by the signaling cascade leading to apoptosis must be upstream of the convergence of TNF and IL-1 signal transduction cascades. In EC, TNF signaling begins with ligand binding to TNFR2, which passes the TNF trimer to TNFR1. Sodium salicylate inhibits R32W TNF-mediated IκBα degradation caused by apoptosis-inducing drugs downstream of TNFR2. The inability of SB203580 to prevent ATK-dependent inhibition of TNF signaling may therefore result from incomplete blocking of p38 activation. Alternatively, these data may suggest a primary role of p38 kinase for responses to sodium salicylate but a secondary role for responses to ATK.

Initiation of Cell Death Reduces TNFR1 Expression and Recruitment of TRADD—The differential effects of apoptosis inducers on TNF- and IL-1-dependent degradation of IκBα suggest that the point of inhibition caused by the signaling cascade leading to apoptosis must be upstream of the convergence of TNF and IL-1 signal transduction cascades. In EC, TNF signaling begins with ligand binding to TNFR2, which passes the TNF trimer to TNFR1. Previous studies in EC have established that R32W TNF TNF mutein protein directly and exclusively signals through TNFR2, bypassing the role of TNFR2 and ligand passing (34). Sodium salicylate inhibits both R32W TNF- and wild type TNF-dependent degradation of IκBα (Fig. 5). Similar results were obtained with all other inducers of apoptosis (data not shown). These data indicate that the point of inhibition of TNF-induced IκBα degradation caused by apoptosis-inducing drugs is downstream of TNFR2.

We next examined the level of expression of cytosolic TNF signaling components. Two h of pretreatment with apoptogenic drugs does not cause a change in the size or quantity of the TRADD, TRAF2, or RIP adaptors proteins (data not shown). A similar approach (i.e. Western blotting) is not useful for evaluation of TNFR1 because the preponderance of this receptor is found in a nonsignaling, Golgi-associated pool (38). However, FACS analysis (Fig. 6 and Table I) show that pretreatment with sodium salicylate reduces surface expression of TNFR1 by at least 50%. Under these same conditions, the effects on surface expression of TNFR2 were not statistically significant. Consistent with a reduction in surface TNFR1, we observed that pretreatment with sodium salicylate reduced TNF-induced recruitment of TRADD to the TNFR1 complex (Fig. 7). These data suggest that the common mechanism

Fig. 4. Effect of the p38 kinase inhibitor SB203580 on the inhibition of TNF-dependent degradation by apoptosis-inducing agents. SB203580 (1, 10 μM) was added to EC for 2 h prior to a further 2-h incubation without or with ATK (50 μM), C6-ceramide (c-6 cer, 50 μM), sodium salicylate (Na Sal, 20 mM), or stauro (100 nM). After this preincubation, TNF (50 units/ml) was added for a further 15 min. Analysis of the degradation of IκBα was determined by Western blotting and densitometry. Data are pooled from three independent experiments and presented as the mean ± S.E. Significance was assessed by a Student’s t test. **, denotes p < 0.01; ***, denotes p < 0.001. □, mock pretreated cells.

Fig. 5. Sodium salicylate inhibits R32W TNF-mediated IκBα degradation. EC were pretreated for 2 h with M199 in the absence (−) or presence (+) of sodium salicylate (Na Sal, 20 mM) before treatment for a further 15 min with R32W TNF (0–10 ng/ml). Cell lysates were analyzed for IκBα degradation by Western blotting.

Fig. 6. Reduction in surface expression of TNFR1 by diverse apoptogenic agents. EC were treated for 2 h with M199 alone (A) or M199 in the presence of ATK (50 μM, B), C6-ceramide (c-6 cer, 50 μM, C), sodium salicylate (Na Sal, 20 mM, D), or stauro (100 nM, E). Cells were stained by indirect immunofluorescence and FACS-analyzed for TNFR1 (dashed line) or irrelevant control mAb (K16/16, solid line) binding.

Fig. 7. Reductive effects of sodium salicylate on TNFR1 expression.
Inhibition of TNF Signaling by Initiation of Apoptosis

TABLE I

Effect of diverse apoptogenic agents on surface expression of TNFR1 and TNFR2

| Treatment          | TNFR1 Mean Fluorescence (arbitrary units) | TNFR2 Mean Fluorescence (arbitrary units) |
|--------------------|------------------------------------------|------------------------------------------|
| Mock               | 5.8 ± 0.8                                | 7.1 ± 0.9                                |
| ATK                | 1.8 ± 0.7**                              | 3.9 ± 2.4                                |
| C6-ceramide        | 2.8 ± 0.7*                               | 5.9 ± 0.5                                |
| Sodium salicylate  | 2.7 ± 0.7*                               | 6.5 ± 0.2                                |
| Stauro             | 2.7 ± 0.2**                              | 5.7 ± 0.9                                |

DISCUSSION

In EC, the activation of NFkB in response to cytokines such as TNF and IL-1 leads to the increased transcription of many genes including those of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 as well as cytokines IL-6 and IL-8 (reviewed in Ref. 40). The activation of these genes is critical in the recruitment of leukocytes into inflammatory reactions. Inhibition of the signal transduction cascade leading to the activation of NFkB is therefore a potential target for novel anti-inflammatory reagents, and many drugs have been reported as displaying such activities. In this study, we confirm that a diverse range of chemical compounds can indeed inhibit TNF signaling. Specifically we observed that ATK, C6-ceramide, sodium salicylate, and stauro all inhibited TNF (and R32W TNF-) but not IL-1-dependent degradation of IkBα. Such apparent specificity has often been interpreted as evidence for pharmacological inhibition of signaling. Significantly, none of these agents has been shown to interact with any of the molecules known to mediate TNF activation of NFkB-inducing kinase, namely TNFR2, TNFR1, TRADD, TRAF2, and RIP. However, we also noted that continued incubation with each of the reagents causes apoptosis, leading us to propose an alternative explanation, namely that the inhibition of TNF-dependent degradation of IkBα results from an early and common step in the signaling cascade leading to apoptosis rather than from direct pharmacological targeting.

The progression of cellular apoptosis is known to be dependent on the activation of a family of aspartic acid proteases termed caspases (reviewed in Refs. 3 and 5), which cleave substrates such as PARP, caspase-activated DNase, nuclear lamin, and focal adhesion kinase (41-44) to cause DNA fragmentation, nuclear condensation, and cytoplasmic shrinkage. Caspases have also recently been implicated in the activation of signal transduction cascades by cleavage and the activation of enzymes such as mitogen-activated protein kinase kinase kinase-1 and cytosolic phospholipase A2 (45, 46). We therefore questioned whether the caspase-dependent cleavage of a TNFR complex component could account for the inhibition of TNF signaling. However, we could not show either that any TNF complex component changed in concentration or molecular weight or that broad spectrum caspase inhibitors zVADfmk or zVADcmk could relieve inhibition due to apoptogenic agents. We next considered the role of the p38 protein kinase, which has been implicated in apoptosis in response to a number of stimuli and in a number of cell types (36, 47-50). Recent evidence has implicated p38 not only in apoptosis (36, 47, 49) but also in the negative regulation of NFkappaB by sodium salicylate (37). As described in previous studies in COS cells (37), the inhibitory effect of sodium salicylate on the TNF signaling pathway could be completely reversed by pretreatment with SB203580. The effect on ATK was less striking, possibly indicative of alternative mechanisms linking apoptosis to loss of signaling.

From our experiments with R32W TNF, we determined that the point of inhibition targeted by apoptosis inducers did not involve TNFR2 or ligand passing and so must lie at or between TNFR1- and NFkB-inducing kinase. All four apoptogenic drugs caused a significant reduction in surface TNFR1 expression, suggesting that an early event in the initiation of programmed cell death results in the decrease of expression of this receptor. To support the interpretation that the decrease in surface TNFR1 accounted for the inhibition of TNF signaling, we considered the effect of sodium salicylate on the TNF-dependent recruitment of TRADD to the receptor complex. Our results demonstrate an early inhibition of the recruitment of this signaling molecule. Thus the common mechanism of TNF signal disruption by apoptogenic drugs appears to be loss of TNFR1. These new data also provide an explanation of a prior obser-

shared by apoptogenic drugs is the reduction in TNFR1 surface expression with consequent inhibition of receptor complex assembly.

Inhibition of TNFR1 Shedding Maintains TNF Signaling.—To determine whether the decrease in surface TNFR1 accounted for the inhibition of TNF signaling, we manipulated surface TNFR1 in the presence of apoptosis-inducing agents using a tert-butyl derivative of Tapi. This agent is an inhibitor of TNF processing and is comparable to that described as an effective inhibitor of the shedding of TNFR1 (39). We observed that the decrease in surface staining of TNFR1 resulting from treatment with apoptosis-inducing agents was paralleled by an increase in soluble TNFR1 detected in culture supernatant by an enzyme-linked immunosorbent assay (data not shown). Pretreatment with Tapi (25 μM) for 30 min prior to the addition of the apoptosis-inducing agents ATK and sodium salicylate maintained surface expression of TNFR1 (Fig. 8A and data not shown). Consistent with this observation, Tapi pretreatment also maintained TNF-dependent degradation of IkBα (Fig. 8B).

However, Tapi did not protect EC from apoptosis in response to either ATK or sodium salicylate (Fig. 8C). This evidence is consistent with our hypothesis that TNFR1 shedding causes loss of TNF signaling in response to apoptosis-inducing agents and suggests that the signaling cascade leading to apoptosis in response to these agents is not dependent on the loss of TNFR1 for its progression.
vation that H$_2$O$_2$, an endogenous mediator of apoptosis, inhibits TNF binding and TNF signaling in EC (51).

We established that the reduction of TNFRI expression results from receptor shedding, which can be effectively blocked by a derivative of the metalloproteinase inhibitor Tapi. Soluble forms of both TNF receptors have been identified in the body fluids of patients with various diseases that are proteolytically derived from the cell surface molecules (52, 53). These soluble receptors can also be detected in the supernatant of cultured cells following both physiological and nonphysiological activation (52, 54–56). A shed receptor can neutralize the bioactivity of circulating TNF by binding to this cytokine, thereby restricting its binding to surface-bound receptors (57, 58). The observation that sodium salicylate is a potent inducer of receptor shedding suggests that the neutralization of circulating TNF may account in part for the action of sodium salicylate as an anti-inflammatory. Shedding of TNFRI is known to be independent of the cytoplasmic region of the receptor (54) but largely dependent on the spacer region between the transmembrane and a conserved extracellular cysteine-rich domains (58). Most critically, residues Val-173 and Lys-174 within the spacer region have been identified as essential for shedding, possibly allowing recognition of the receptor by a metalloproteinase that ultimately cleaves the receptor from the surface (58, 59). To date, the protease that specifically cleaves TNFRI from the surface of the cell has not been identified, and therefore it is not known how the activity of the protease is regulated. Some evidence suggests that the shedding of both TNF receptors may be dependent on a phosphorylation event (60, 61). Such a mechanism could be implicated in the shedding of TNFRI induced by apoptogenic agents when p38 kinase is activated.

In conclusion, our observations provide a toxicological rather than direct pharmacological explanation for the effects of many structurally diverse agents on TNF signaling. Evidence points to a loss of TNFRI from the cell surface as the key step involved in apoptosis-induced inhibition of TNF signal transduction. We speculate that down-regulation of TNF signaling through this mechanism may serve to limit inflammation during apoptosis.

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REFERENCES

1. Tartaglia, L. A., and Goeddel, D. V. (1992) Immunol. Today 13, 151–153
2. Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadam, E., Leitner, O., and Wallach, D. (1990) J. Biol. Chem. 265, 14497–14504
3. Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A., Jr., and Goeddel, D. V. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9292–9296
4. Haridas, V., Durnay, B. G., Natarajan, K., Heller, R., and Aggarwal, B. B. (1998) J. Immunol. 160, 3152–3162
5. Tartaglia, L. A., Pennica, D., and Goeddel, D. V. (1993) J. Biol. Chem. 268, 18542–18549
6. Pinkard, J. K., Sheehan, K. C., and Schreiber, R. D. (1997) J. Biol. Chem. 272, 10784–10789
7. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
8. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
9. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996) Immunity 4, 387–396
10. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Chai, Y. (1997) Immunity 7, 703–713
11. Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
12. Kelliher, M. A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B. Z., and Leder, P. (1998) Immunity 8, 297–303
13. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
14. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) Proc. Natl Acad. Sci. U. S. A. 94, 9792–9796
15. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 385, 548–554
16. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Mann, M., Mann, P., and Rao, S. (1997) Science 278, 860–866
17. Wronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) Science 278, 860–869
18. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
19. Verma, I. M., Stevenson, J. K., Schwartz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735
Inhibition of TNF Signaling by Initiation of Apoptosis

13649

20. Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M., and Verma, I. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12740–12744

21. Traenckner, E. B., Wilk, S., and Bacacer, P. A. (1994) EMBO J. 13, 5433–5441

22. Greenfeder, S. A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R. A., and Ju, G. (1995) J. Biol. Chem. 270, 13757–13765

23. Wescie, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) Immunity 7, 837–847

24. Wescie, H., Korherr, C., Kracht, M., Falk, W., Resch, K., and Martin, M. U. (1997) J. Biol. Chem. 272, 7727–7731

25. Cao, Z., Henzel, W. J., and Gao, X. (1996) Science 271, 1128–1131

26. Read, M. A., Whiteley, M. Z., Williams, A. J., and Collins, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 503–512

27. Read, M. A., Whiteley, M. Z., Collins, T., and Cobert, J. S. (1994) J. Exp. Med. 179, 503–512

28. De Luca, L. G., Johnson, D. R., Whiteley, M. Z., Collins, T., and Pober, J. S. (1994) J. Biol. Chem. 269, 19193–19196

29. Lane, T. A., Lamkin, G. E., and Wancewicz, E. V. (1990) J. Biol. Chem. 265, 5830–5833

30. Stuhlmeier, K. M., Kao, J. J., and Bach, F. H. (1997) J. Biol. Chem. 272, 24679–24683

31. Slowik, M. R., De Luca, L. G., Min, W., and Pober, J. S. (1996) Circ. Res. 79, 736–747

32. Pierce, J. W., Read, M. A., Ding, H., Luscinskas, F. W., and Collins, T. (1996) J. Immunol. 156, 3961–3969

33. Laemmli, U. K. (1970) Nature 227, 680–685

34. Slowik, M. R., De Luca, L. G., Fiers, W., and Pober, J. S. (1993) Am. J. Pathol. 143, 1724–1730

35. Cohen, G. M. (1997) Biochem. J. 326, 1–16

36. Schwenger, P., Bellotta, P., Viertler, I., Basilo, A., Skolnik, E. Y., and Vilcek, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 94, 2869–2873

37. Schwenger, P., Alpert, D., Skolnik, E. Y., and Vilcek, J. (1998) Mol. Cell. Biol. 18, 78–84

38. Bradley, J. R., Thiru, S., and Pober, J. S. (1995) Am. J. Pathol. 146, 27–32

39. Nimmer, J. D., Dusek, J., Hahn, D. M., Otten-Evans, C., Aderka, D., Kolesnik, R. N. (1992) Arthritis Rheum. 35, 1160–1169

40. Brakebusch, C., Nophar, Y., Kemper, O., Engelmann, H., and Wallach, D. (1992) EMBO J. 11, 943–950

41. Lantz, M., Bjornberg, F., Olsson, I., and Richter, J. (1994) J. Immunol. 152, 1369–1375

42. Baeuerle, P. A., and Huggett, J. (1996) Biochem. Biophys. Res. Commun. 221, 1273–1281

43. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1996) Nature 381, 43–50

44. Slowik, M. R., De Luca, L. G., Fiers, W., and Pober, J. S. (1993) J. Exp. Med. 178, 1362–1369

45. Brakebusch, C., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1999) Cell 90, 315–325

46. Verheij, M., Rose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Saba, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Piers, Z., and Kolesnick, R. N. (1996) Nature 380, 75–79

47. Lantz, M., Bjornberg, F., Olsson, I., and Richter, J. (1994) J. Immunol. 152, 1369–1375

48. Brakebusch, C., Nophar, Y., Kemper, O., Engelmann, H., and Wallach, D. (1992) EMBO J. 11, 943–950

49. Lantz, M., Bjornberg, F., Olsson, I., and Richter, J. (1994) J. Immunol. 152, 1369–1375

50. Gullberg, U., Baysal, B., Kaufmann, S. H., and Earnshaw, W. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9042–9046

51. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1999) Cell 90, 315–325

52. Wissing, D., Mootza, H., Egeblad, M., Poirier, G. G., and Jaattela, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5073–5077

53. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1999) Cell 90, 315–325

54. Brakebusch, C., Nophar, Y., Kemper, O., Engelmann, H., and Wallach, D. (1992) EMBO J. 11, 943–950

55. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1996) Nature 381, 43–50

56. Verheij, M., Rose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Saba, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Piers, Z., and Kolesnick, R. N. (1996) Nature 380, 75–79