



TNF down-regulation of receptor tyrosine kinase-dependent mitogenic signal pathways as an important step in cytostasis induction and commitment to apoptosis of Kym-1 rhabdomyosarcoma cells

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Abstract

Growth of Kym-1 rhabdomyosarcoma cells depends on endogenous receptor tyrosine kinase signals activated by insulin and insulin-like growth factors (IGF), as revealed from enhancement of proliferation by insulin and IGF-1 and cytostatic action of inhibitors of IR/IGFR kinases. Depending on the presence or absence of the caspase inhibitor z-VAD-fmk, TNF induced full growth arrest or apoptosis, respectively, indicating dominance of TNF over mitogenic signal pathways in Kym-1 cells. In accordance with a caspase-independent cytostatic action, TNF downregulated IR kinase activity and caused a profound inhibition of downstream mitogenic signals including the MAPK cascade and STAT5, key pathways of proliferation and cell survival. Removal of z-VAD-fmk after 24 h induced rapid cell death in the absence of TNF. The inhibition of survival signals concomitant with persisting proapoptotic signals may tip the balance towards an irreversible commitment of the cell to apoptosis that becomes apparent upon relief of suppression of effector caspases. *Cell Death and Differentiation* (2000) 7, 955–965.

Keywords: TNF signal crosstalk; MAPK pathway; STAT5; growth arrest; apoptosis

Abbreviations: EMSA, electrophoretic mobility shift assay; GLUT4, glucose transporter 4; IR β , insulin receptor β chain; IGFR, insulin-like growth factor receptor; IRS-1, insulin receptor substrate 1; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; NIDDM, non-insulin-dependent diabetes mellitus; PIP₃, phosphatidylinositol-3,4,5-trisphosphate; TNF/R, tumor necrosis factor/receptors; RTK, receptor tyrosine kinases; STAT5, signal transducer and activator of transcription 5; z-VAD-fmk, z-Val-Ala-DL-Asp-fluoromethylketone

Introduction

Tumor necrosis factor (TNF) is an important mediator of inflammation and inducer of cell death in a variety of cell types via TNF-R1 and TNF-R2-dependent engagement of multiple signal pathways, typically resulting in NF- κ B and JNK activation in virtually all cells, as well as induction of caspase cascades in cells sensitive to apoptosis (for reviews see^{1,2}). Due to the potentially simultaneous activation of pro- and antiapoptotic signal pathways by TNF, the cellular context, i.e. the qualitative and quantitative constitution with the respective signal components, determines the cellular fate in response to TNF. In addition to the direct activation of proinflammatory and apoptotic signal cascades, it becomes increasingly evident that TNF signals also affect other signal cascades and, in particular, interfere with several receptor tyrosine kinase-mediated activities. For example, the TNF crosstalk with insulin signal pathways has received much attention in recent years, because a link and potential causal relationship to the development of insulin resistance, a widespread pathophysiological condition leading to NIDDM, has been proposed.³ This is based on both *in vitro* cell models with adipocytes and hepatocytes, where TNF interfered with insulin-induced IR activation,^{4,5} as well as *in vivo* models of genetic or dietary development of obesity and diabetes, showing amelioration of insulin resistance in TNF- or TNFR-k/o mice.⁶ The responsible molecular mechanisms of this TNF crosstalk are not yet understood and may operate at various levels of the insulin signal cascade, probably involving a down-regulation of the glucose transporter GLUT4 itself,⁷ inhibition of IRS-1 function,^{5,8,9} and direct inhibition of IR β kinase activity.^{10–12} However, the partial inhibition of IR β kinase activity appears by itself not sufficient to explain reduced glucose uptake, as in muscle cells the latter is not affected by TNF despite a down-regulation of IR β kinase and immediate downstream substrates.¹³ In addition to the prominent IR signal transducer IRS-1, STAT5b was recently recognized as a direct substrate.^{12,14,15} Both, IRS-1 and STAT5b are considered as important regulators of proliferative and antiapoptotic responses.^{16–22} IRS-1 proteins act as interfaces between stimulated receptors and downstream signaling proteins with SH2 (src homology 2) domains such as Grb2 and Shc, which activate the MAP kinase (MAPK) cascade^{23–25} and PI 3-kinase (phosphatidylinositol-3-OH kinase), whose product PIP₃ (phosphatidylinositol-3,4,5-trisphosphate) is an activator of Akt/PKB (protein kinase B). Interestingly, not only Akt/PKB, which function to promote cell survival by inhibiting apoptosis at various levels,^{26,27} but also STAT5 are constitutively active in an apoptosis-resistant cell line,²² suggesting that besides Akt/PKB, STAT5 and STAT5

dependent genes also might play a role in the prevention of apoptosis. Accordingly, TNF mediated crosstalk with insulin signaling could extend beyond an interference with insulin's immediate metabolic effects causing insulin resistance and affect signal pathways regulating the transcriptional program and cellular functions related to growth control and survival.

To further substantiate at a molecular level the extent of the TNF crosstalk with mitogenic RTK-mediated signals induced by insulin and to analyze the functional consequences with respect to growth control and apoptosis sensitivity, we have studied a human rhabdomyosarcoma cell line, Kym-1. Although these cells display an endogenous activation of the related insulin-like growth factor receptor (IGFR), which shares the activation of several intracellular signal pathways, in particular IRS-1 activation, with the IR,²⁸ Kym-1 cells are responsive to exogenous insulin treatment with respect to IRS-1 activation.¹⁵ As Kym-1 cells are also highly sensitive to TNF and rapidly undergo apoptosis,^{29,30} the insulin signal pathways potentially affected by TNF were investigated under conditions of blocking the apoptotic effector phase by caspase inhibitors. The data obtained show an early inhibition of insulin-induced mitogenic and potentially antiapoptotic signals, resulting in a complete growth arrest during a two day culture. In parallel, within 24 h, cells become irreversibly committed to death; upon removal of the broad spectrum caspase inhibitor z-VAD-fmk, cells undergo apoptosis in the absence of exogenous TNF. The rapid, profound and lasting inhibition of mitogenic/antiapoptotic signals could be an essential step towards progression to apoptosis.

Results

Kym-1 rhabdomyosarcoma growth depends on functional insulin/IGF signal pathways

The functionality of the IR signaling was first verified by Western blot analyses of tyrosine phosphorylation of immunoprecipitated IR β and IRS-1. Upon insulin treatment of Kym-1 cells, both, IR β and IRS-1, were rapidly tyrosine phosphorylated reaching maximum phosphorylation within 1 min of stimulation and a subsequent slow decrease with still elevated phosphorylation of both molecules detectable after 60 min (Figure 1A). Of note, IRS-1 already showed weak, but clearly discernable tyrosine phosphorylation in untreated cells, indicating constitutive IRS-1 activation under standard cell culture conditions, probably due to endogenous IGFR signaling, a major mitogenic signal pathway of rhabdomyosarcoma cells.³¹ Although growth of Kym-1 cells was largely independent of addition of insulin and IGF-1 to a complete culture medium, under reduced serum conditions the mitogenic effect of both insulin and IGF-1 became apparent (Figure 1B). Insulin treatment resulted in an increase in thymidine incorporation, which could be blocked by the tyrosine kinase inhibitors Tyrphostin AG1024 (Figure 1C) and HNMPA[AM]₃ (data not shown) below the basal level of thymidine incorporation of untreated cells. As these substances are considered to selectively inhibit IR and IGFR, but not EGFR kinase^{32,33} a role of a constitutively active IGFR signal pathway for proliferation of Kym-1 cells is suggested.

Both inhibitors caused a dose-dependent inhibition of proliferation (Figure 1D, E), but not induction of apoptosis (as revealed from microscopical examination as well as propidium iodide staining, data not shown) within the observation period. In fact, at the highest dose employed, a complete growth arrest was achieved as evident from the MTT signal obtained from an aliquot of freshly seeded cells stained directly after attachment to culture dishes (Figure 1D, E), asterisk and dotted line). Together, these data suggest an essential participation of IR/IGFR induced signals to the proliferative capacity of Kym-1 cells.

TNF down-regulates insulin-induced activation of mitogenic signal pathways in Kym-1 cells

Recently, a negative regulatory crosstalk between insulin and TNF signaling cascades has been identified, a hallmark being the inhibition of insulin-induced, GLUT4-mediated glucose uptake in adipocytes,⁴ whereas in muscle cells, this crosstalk does apparently not affect glucose transport.¹³ Kym-1 cells are TNF-responsive as evident from rapid, but transient NF- κ B activation (Figure 2A), and the later induction of apoptosis without conditioning of the cells by protein synthesis inhibitors^{29,30} (Figure 4A). In accordance with previous results obtained in other cell models^{4,5,11,12} a 2 h TNF pretreatment of Kym-1 cells resulted in a typical amelioration of the subsequent insulin response, with a partial (30–50%) reduction of IR β kinase activity (Figure 2B). In these cells, significant TNF mediated caspase activation can only be revealed after approximately 3 h of TNF treatment.³⁰ Nevertheless, to ensure that low levels of active caspases did not influence early events in the signal crosstalk between TNF and insulin, z-VAD-fmk, an effective, broad spectrum inhibitor of caspases,³⁴ was added throughout the TNF pretreatment and subsequent stimulation period. Immunoblot analyses verified down-regulation of IR β kinase activity by TNF also in the presence of z-VAD-fmk (Figure 2C), indicating caspase independence of this inhibitory crosstalk. As a consequence of reduced insulin receptor kinase activity, tyrosine phosphorylation of the direct substrates IRS-1 and STAT5b was reduced to the same or greater extent as the IR without decrease in protein levels (Figure 2D, E). Since STAT5b tyrosine phosphorylation is an absolute prerequisite for nuclear translocation and transcriptional activity, EMSA analyses were performed to investigate the functional significance of reduced tyrosine phosphorylation of STAT5b. Interestingly, upon TNF pretreatment of Kym-1 cells, insulin-induced STAT5b translocation was completely blocked (Figure 2F). These data already indicate that the crosstalk of TNF also affects insulin-induced transcriptional programs, which was further corroborated by studying the influence of TNF on insulin's mitogenic activities via IRS-1 and Shc, which converge at the level of Raf-1 as the upstream activator of the MAPK cascade.

Upon TNF pretreatment of Kym-1 cells, insulin-mediated induction of the MAPK cascade was significantly reduced. This was revealed from direct and linked Raf-1 kinase assays, using c-Raf-1 immunoprecipitations from stimulated Kym-1 cells and GST-MEK1 (Figure 2G, middle panel), or GST-MEK1 and kinase-negative GST-Erk1 (Figure 2G,

lower panel) as substrates for *in vitro* kinase assays. TNF pretreatment did not influence protein levels of c-Raf-1

(Figure 2G, upper panel). Further, Erk2 immunoprecipitates and Erk2 kinase assays using MBP as a substrate verified

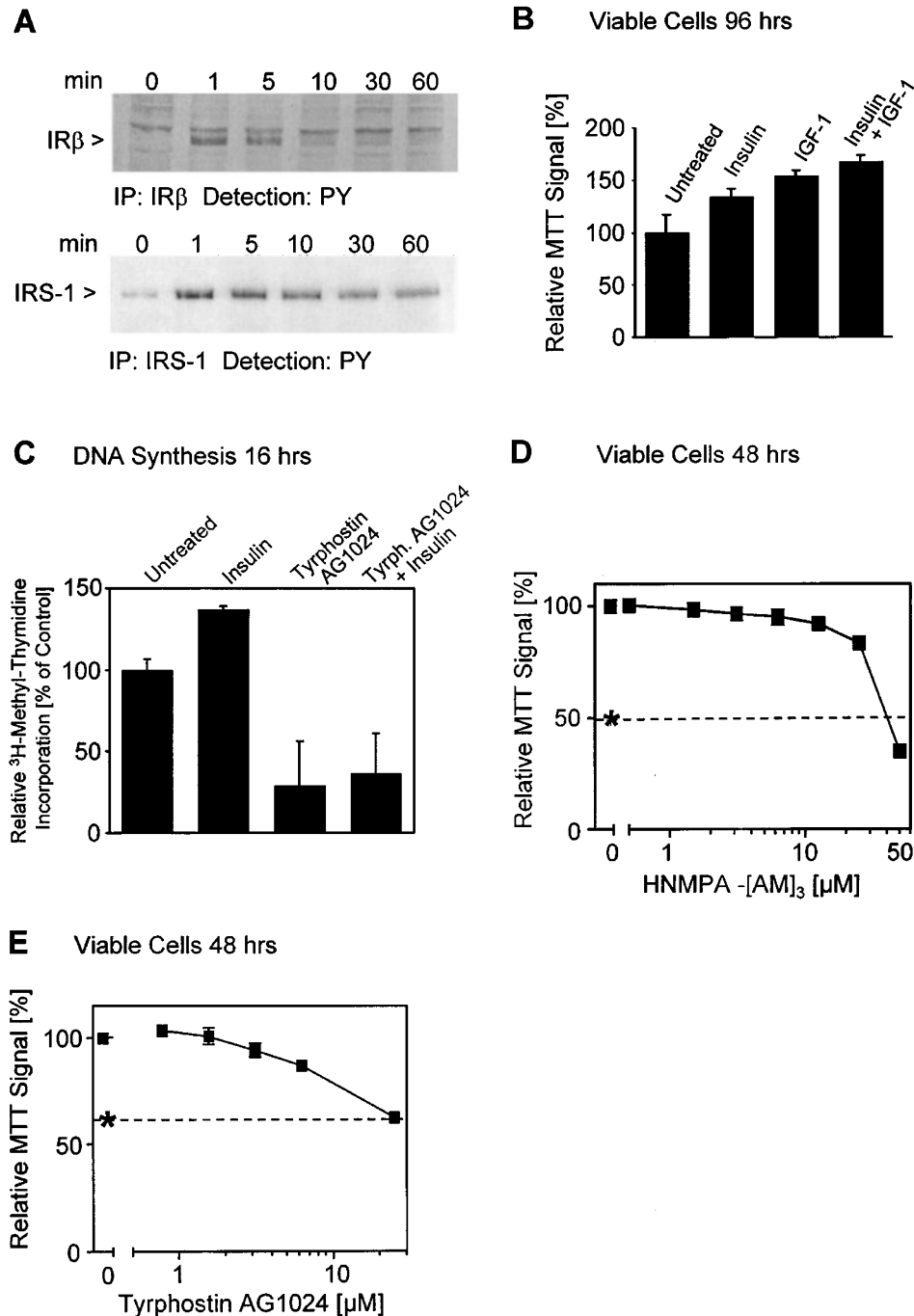


Figure 1 Dependence of Kym-1 cell proliferation on insulin/IGFR kinase activity. **(A)** Kinetics of insulin induced tyrosine phosphorylation of IRβ and IRS-1. 5×10^5 synchronized Kym-1 cells were serum-starved for 2 h and stimulated with insulin (100 nM) for indicated times. Cells were lysed in lysis buffer, and IRβ or IRS-1 were immunoprecipitated. The samples were separated on SDS-PAGE, transferred to nitrocellulose and stained with PY-specific antibodies (PY99). **(B)** Proliferation of Kym-1 is enhanced by insulin and IGF-1. Kym-1 cells (2000 per well; 96-well plate) were treated with insulin (10 ng/ml) or/and IGF-1 (10 ng/ml) under reduced serum conditions (0.5% FCS). After 96 h, the number of viable cells was determined with the MTT kit. **(C)** Insulin-induced enhanced proliferation of Kym-1 is abrogated by insulin/IGF-specific RTK inhibitors. Kym-1 cells were either left untreated or cultivated with insulin, Tyrphostin AG1024 (40 μM), or insulin and Tyrphostin AG1024 in cell culture medium containing 0.5% FCS, and pulsed for 16 h with ³H-Thymidine. **(D, E)** Cytostatic effects of HNMPA-[AM]₃ and Tyrphostin AG1024. Kym-1 cells were incubated with indicated concentrations of HNMPA-[AM]₃ **(D)** or Tyrphostin AG1024 **(E)**. After 48 h, living cells were determined with the MTT kit as described in Material and Methods. Asterisks and dotted line indicate the MTT signal obtained from an aliquot of cells directly after seeding and attachment. The MTT value of untreated control cells obtained after 48 h culture was set 100%. All experiments have been performed three times with similar results

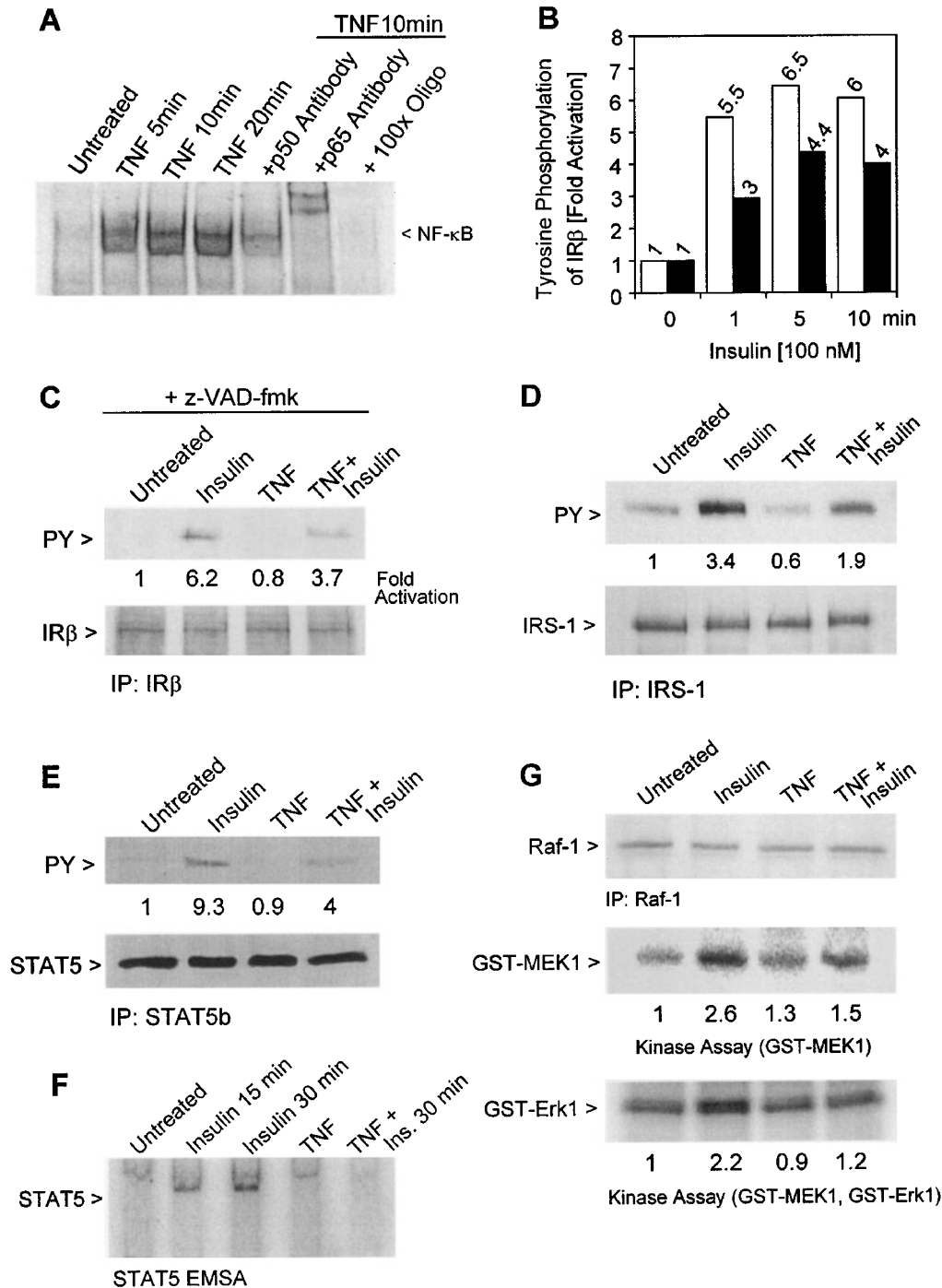


Figure 2 TNF signaling and crosstalk with insulin-induced mitogenic signal pathways in Kym-1 cells. **(A)** Activation of p50/p65 NF- κ B homo/heterodimers by TNF. 10^6 Kym-1 cells were serum-starved for 2 h and stimulated with TNF (50 ng/ml) for the indicated times. Nuclear extracts were prepared, EMSA and 'supershift' EMSA were performed as described in Materials and Methods. The specificity of the NF- κ B/oligonucleotide interaction was determined by competition with an excess of 100-fold non radio-labeled oligonucleotide. **(B)** Time course of TNF-induced inhibition of IR β . 5×10^6 Kym-1 cells were serum-deprived and preincubated for 2 h without or with TNF (50 ng/ml). After stimulation with insulin (100 nM) for indicated times cells were lysed in lysis buffer and IR β was immunoprecipitated. The immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose and Western blotting with phosphotyrosine specific (PY99) and IR β specific antibody was performed. Densitometric evaluation of blots was done with a phosphorimager and fold activation of the IR kinase of insulin-treated cells relative to untreated cells is shown above the bars (white bars=insulin stimulation; black bars=TNF pretreatment before insulin stimulation). **(C)** Inhibition of insulin-induced IR β activity by TNF in presence of z-VAD-fmk. Kym-1 cells were treated as in B plus addition of z-VAD-fmk (20 μ M) during TNF pretreatment. Insulin stimulation (100 nM) was for 5 min, cell lysates, immunoprecipitation and Western blotting were done as in B. **(D)** Inhibition of insulin-induced tyrosine phosphorylation of IRS-1 by TNF. IRS-1 was immunoprecipitated from 5×10^6 Kym-1 cells pretreated as in B, upon insulin stimulation (100 nM) for 5 min. The blot was immunostained with antibodies against PY (PY99) or IRS-1. **(E, F)** Inhibition of insulin-induced activation of STAT5 by TNF in Kym-1 cells. **(E)** STAT5 was immunoprecipitated from 5×10^6 Kym-1 cells pretreated as in B and stimulated with insulin (100 nM) for 30 min. The blot was stained with PY (4G10, upper panel) or STAT5b antibodies (G2, lower panel). **(F)** EMSA analysis of STAT5 activation. 10^6 Kym-1 cells were pretreated as in E and stimulated with insulin (100 nM) for 15 or 30 min, all in the presence of 20 mM

that insulin-induced Erk2 activity was also completely abolished upon TNF pretreatment (data not shown).

Thus TNF inhibits insulin/IGF-1 mediated growth signals within a time period, at which caspase activation is not yet detectable, and where cytotoxic actions can be rescued by the addition of caspase inhibitors.³⁰ In order to investigate whether these inhibitory effects are only transient or persist over a long time period, long term (up to 48 h) TNF treatment of Kym-1 rhabdomyosarcoma cells was performed in the presence of the broad spectrum caspase inhibitor z-VAD-fmk in order to prevent apoptosis. The data obtained revealed a lasting, profound inhibition of tyrosine

phosphorylation at both insulin receptor and IRS-1 levels (Figure 3A, C). A similar degree of inhibition could be obtained when the cells were cultured in the presence of Tyrphostin AG1024 before insulin stimulation (Figure 3B, D). In both cases no changes in the protein levels of IR and IRS-1 were observed.

TNF induces growth inhibition and morphological changes when apoptosis is blocked

Kym-1 cells are highly sensitive to TNF induced apoptosis, with nearly complete cell death already at 100 pg/ml of TNF

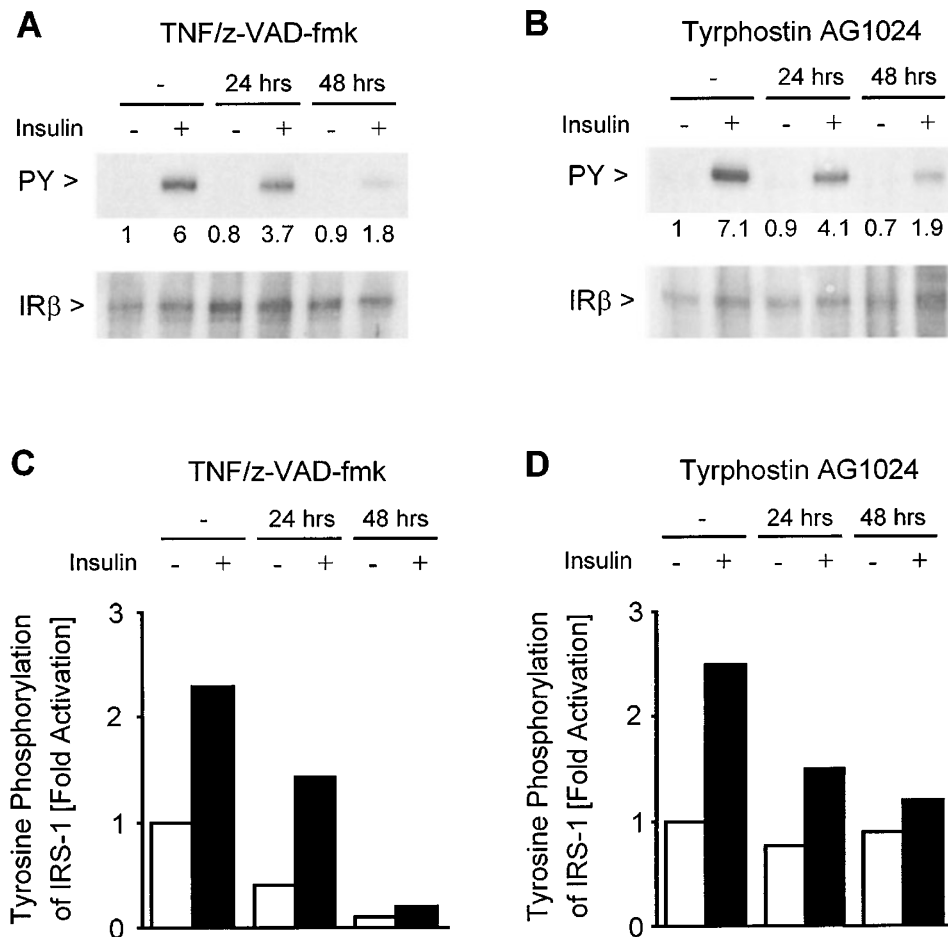


Figure 3 Inhibition of insulin signals after long-term treatment with TNF/z-VAD-fmk or Tyrphostin AG1024. Kym-1 cells were pretreated with TNF (50 ng/ml) and z-VAD-fmk (20 μ M), or with Tyrphostin AG1024 (25 μ M) for 24 or 48 h. Insulin stimulation (100 nM) was for 5 min (**A,B**) IR β was immunoprecipitated from 5×10^6 Kym-1 cells and Western blotting against PY (4G10) was performed (upper panel). The blot was stripped and re-probed against the IR β -chain (lower panel). (**C,D**) IRS-1 was immunoprecipitated from 5×10^6 Kym-1 cells. Western blotting with antibodies against PY (4G10), and re-probing of the blot against IRS-1 was performed. Basal IRS-1 tyrosine phosphorylation was set as 1. All experiments have been performed three times with similar results

sodium vanadate. Nuclear extracts were prepared and STAT5 EMSA was performed. (**G**) Inhibition of insulin-induced Raf-1 activation by TNF. 5×10^6 Kym-1 cells were kept in 0.5% FCS containing medium over night. Prior to the stimulation they were pretreated as in **B** and then stimulated with insulin (100 nM) for 1 min. Raf-1 kinase was immunoprecipitated and kinase assays were performed. Upper panel shows immunoprecipitated Raf-1 kinase, indicating equal amounts of protein in the precipitates. Lower two panels show a direct kinase assay of immunoprecipitated Raf-1 kinase using inactive GST-MEK1 as a substrate (middle panel) and a linked kinase assay with GST-MEK1 and kinase dead GST-Erk1 as substrate (lower panel). The samples were run on SDS-PAGE and transferred to nitrocellulose and analyzed with a phosphorimager. All experiments have been performed three times with similar results

(Figure 4A). In order to reveal functional consequences of the TNF insulin crosstalk independent of concomitantly ongoing apoptotic processes, that are likely to blur other intracellular signals and cellular responses, culture and stimulation of the cells was done in the presence of z-VAD-fmk. Under these

conditions, a cytostatic action of TNF became apparent, reaching full growth arrest at around 10 ng/ml (Figure 4A). Although in the same cell line, the TNF dose is significantly higher than that required for induction of apoptosis, it is just around the concentration necessary to reach saturation

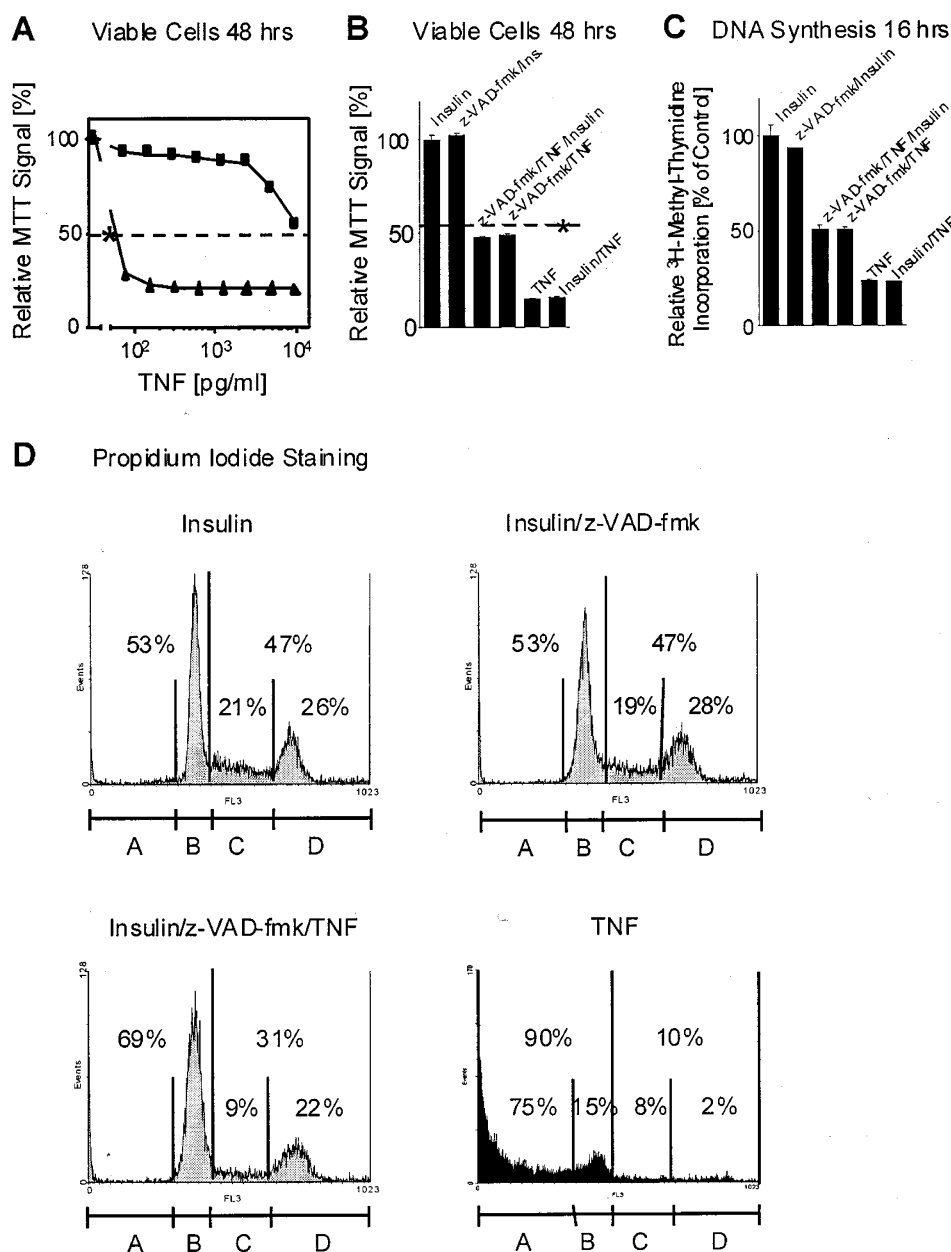


Figure 4 Cytostatic action of TNF in presence of z-VAD-fmk. **(A)** Differential sensitivity to the cytotoxic and cytostatic action of TNF. 10^5 Kym-1 cells were incubated for 48 h with the indicated concentrations of TNF in the presence (filled squares) or absence (filled triangles) of z-VAD-fmk (20 μ M). Viable cells were determined by MTT assay. The MTT signal of an aliquot of cells stained directly after attachment (asterisk and dotted line) is shown as a reference to the proliferation of untreated cells after 48 h, the latter value was arbitrarily set as 100%. **(B,C)** Insulin costimulation does not prevent TNF's cytotoxic or cytostatic action. **(B)** MTT assay. Kym-1 cells were treated for 48 h as indicated (insulin 100 nM; TNF 50 ng/ml; z-VAD-fmk 20 μ M) in cell culture medium containing 10% FCS. Asterisk and dotted line indicate the MTT signal of the cells at onset of culture. **(C)** Thymidine incorporation. After attachment, Kym-1 cells were treated as indicated (insulin 100 nM; TNF 50 ng/ml; z-VAD-fmk 20 μ M) for 16 h in the presence of 3 H-thymidine according to Materials and Methods. **(D)** Cell cycle analyses of propidium iodide stained Kym-1 cells treated for 8 h either with insulin (100 nM) only, insulin (100 nM)/z-VAD-fmk (20 μ M), insulin (100 nM)/z-VAD-fmk (20 μ M)/TNF (50 ng/ml) or TNF (50 ng/ml) only. Propidium iodide staining was performed as described in Materials and Methods. The samples were analyzed by flow cytometry. (area A: hypoploid nuclei indicating apoptotic cells; area B: cells in G0/G1 phase; area C: cells in S phase; area D: cells in G2/M phase). Shown is one representative experiment out of five with similar results

binding to membrane expressed TNF-R1,³⁵ the receptor responsible for induction of apoptosis³⁶ and signal crosstalk with insulin.¹² Insulin did neither prevent TNF's apoptotic activity on Kym-1 cells nor, when apoptosis was blocked by z-VAD-fmk, its cytostatic action, as revealed from MTT assays after 48 h (Figure 4B) and thymidine incorporation assays after 16 h (Figure 4C). The dominant cytostatic action of TNF thus resembles the action of the RTK inhibitors (Figure 1) and is in full accordance with the biochemical data (Figures 2 and 3), showing TNF mediated inhibition of insulin-induced mitogenic signal cascades. TNF-mediated induction of cytostasis, in the presence of z-VAD-fmk, was independently confirmed by cell cycle analyses using flow cytometry of propidium iodide stained Kym-1 cells after 8 h of treatment. These experiments provided clear evidence for a growth arrest, but not apoptosis, by accumulation of insulin/TNF/z-VAD-fmk treated cells in G0/G1 (area B) in the absence of hypoploid cells (area A) (Figure 4D, lower left panel),

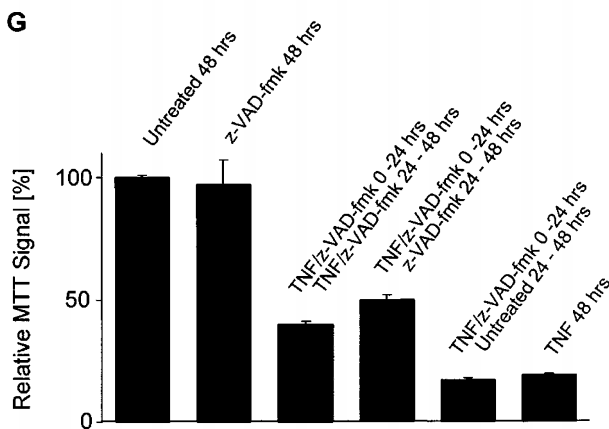
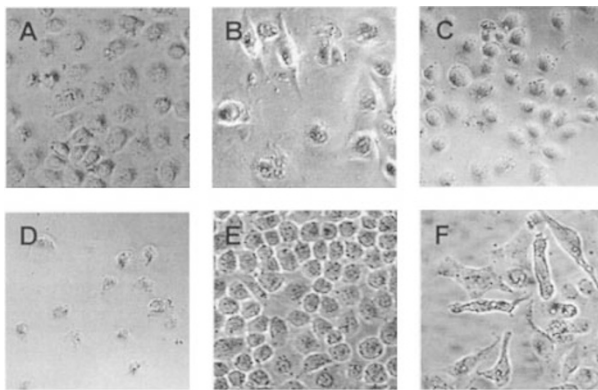


Figure 5 TNF induces morphological changes in Kym-1 cells when apoptosis is blocked by caspase inhibitors. Kym-1 cells were incubated 48 h (A–D) and 96 h (E,F) either with 20 μ M z-VAD-fmk plus 50 ng/ml TNF (B and F), with 20 μ M z-VAD-fmk (C), or left untreated (A and E). In (D), after 24 h the culture medium containing TNF+z-VAD-fmk was changed into standard growth medium. Shown are phase contrast pictures (final magnification $\times 40$). (G) Kym-1 cells were preincubated for 24 h with z-VAD-fmk (20 μ M) plus TNF (50 ng/ml), as controls with z-VAD-fmk or TNF alone, or were left untreated. After 24 h the culture medium was changed into standard growth medium containing indicated agents. After additional 24 h a MTT assay was performed. The MTT signal obtained from untreated cells (48 h) served as reference and was set 100%. The experiments have been performed three times with similar results

compared to non TNF treated controls (Figure 4D, insulin and insulin/z-VAD-fmk, upper panels, respectively). Interestingly, the most prominent changes were observed in the fraction of cells entering the S-phase of cell cycle, with a reduction from 20 to 9%, whereas the number of cells in G2/M didn't drop so much (27 and 22% for controls and TNF, respectively). The same result was obtained when cell cycle analysis was performed at 96 h of combined TNF/z-VAD-fmk treatment (data not shown). This suggests that TNF affects early and late restriction points in cell cycle, namely entry into S-phase, as well as entry into and/or exit from mitosis.

Upon persisting blockade of caspases by z-VAD-fmk, prolonged TNF treatment of Kym-1 rhabdomyosarcoma cells resulted in gross changes of the cell morphology, detectable at 48 h of culture (Figure 5B), but more prominent at 96 h (Figure 5F) in comparison to untreated (Figure 5A, E), or z-VAD-fmk treated control cells (Figure 5C). At the latter time point, TNF treated cells showed, in comparison to controls, a stretched-out irregular shape and an approximately doubled size. This morphological alteration is, in contrast to the parallel, irreversible commitment to apoptosis, a rather slow process that is reversible at least during the first 24 h of treatment. Thus, growth inhibited Kym-1 cells apparently resumed growth upon removal of TNF when z-VAD-fmk treatment was continued (Figure 5G), whereas upon simultaneous removal of TNF and the caspase inhibitor z-VAD-fmk from the culture after 24 h, the cells undergo apoptosis without further exogenous signals (Figure 5D, G), indicating the latent proapoptotic state of the cell.

Discussion

We here show that the rhabdomyosarcoma cell line Kym-1 is a suitable model to study signal crosstalk of cell death and cell growth inducing ligands. *In vitro* proliferation of this cell line depends on endogenous and exogenously triggered activity of insulin and IGF receptor tyrosine kinases, evident from cytostasis induced by inhibitors of these kinases (Figure 1). On the other hand, Kym-1 cells are highly sensitive to TNF,²⁹ with rapid, but transient NF- κ B activation and, upon persistent TNF exposure for several hours, induction of apoptosis.³⁰ The TNF signal crosstalk with insulin occurs early, before caspases are activated and other, irreversible apoptotic processes ensue. TNF induced signals affect the IR kinase activity itself and, to a greater extent, several downstream targets of the IR along the mitogenic and survival signal pathways of insulin, specifically ERKs as well as STAT5b (Figure 2). Both, STAT5 and MAPK are considered as typical positive regulators of cell cycle progression. As for STAT5, nuclear translocation is also a prerequisite for MAPK to promote entry into the cell cycle.^{37,38} Therefore, our data provide a new perspective to mechanisms of TNF crosstalk with mitogenic signaling cascades that becomes manifested at the transcriptional level due to preventing activation of nuclear MAPK targets and of STAT5 regulated genes. Accordingly, we suggest that inhibition of both, STAT5 and MAPK activation contributes to the cytostatic action of TNF. By a block of the execution of apoptosis with the specific caspase inhibitor z-VAD-fmk, we were further able to

investigate the long term consequences of TNF's crosstalk with these mitogenic signal pathways. A persistent inhibition of the IR β kinase activity by TNF (Figure 3), as well as a potent cytostatic activity of TNF on Kym-1 cells similar to RTK inhibitors (Figure 4) became apparent under these conditions. Thus, the close correlation of the observed TNF-mediated cytostasis in Kym-1 cells and TNF-mediated inhibition of signal pathways considered as important positive regulators of the cell cycle suggest a causal relationship.

Several mechanisms have been previously described influencing the activation status of the insulin receptor β -chain. Serine phosphorylation of IR β , as well as of IRS-1 are discussed to induce insulin resistance, and can occur within few minutes after TNF treatment.^{5,8–10,53} In Kym-1 cells treatment with TNF (1 min to 2 h) following stimulation with insulin did not lead to co-immunoprecipitation of IRS-1 in IR β immunoprecipitates (data not shown); the latter could be taken as indication of a rapid inhibition of IR β by serine phosphorylated IRS-1, as it was described before.^{5,8} Due to lack of experimental evidence supporting such a mechanism in Kym-1 cells, we conclude that another mechanism i.e. the activation of tyrosine phosphatases might be responsible for the reduction in tyrosine phosphorylation. It was shown before that the tyrosine phosphatase inhibitor orthovanadate can suppress the inhibitory effects of TNF on insulin signaling cascades.⁵⁴ Therefore, it is reasonable to assume that in our cellular system the primary inhibitory effect occurs directly at the insulin receptor β -chain. The direct substrates of the insulin receptor, IRS-1 and STAT5b, are both implicated in proliferative signaling.^{16–19} A TNF mediated down-regulation of IRS-1 signals appears of significance, as IRS-1 takes a central position converging both survival and mitogenic signals, in particular activation of PI 3-kinase, Akt-1 (PKB) and MAPK, which mediate proliferative as well as antiapoptotic signals.^{26,27,39} The insulin-mediated activation of STAT5b and its inhibition by TNF in Kym-1 cells is in accordance with recent own and other data demonstrating that STAT5 is directly phosphorylated by the insulin receptor kinase¹⁴ and that TNF induced inhibition of insulin-mediated STAT5b tyrosine phosphorylation results in complete loss of STAT5b nuclear translocation.¹² The physiological function of STAT5 is apparently complex and not fully elucidated yet.^{37,40} However, an involvement of STAT5 in survival and proliferation of different cell types is emerging from several studies.^{17,18,21,41} STAT5 is capable to act in concert with other transcription factors,⁴² and for different STAT family members, including STAT5a, an interaction with MAPK signaling pathways in RTK and insulin signaling were implicated.^{43,44} Moreover, in several cells the disruption of the STAT, as well as the Raf-1/MAP kinase pathways blocks survival factors targeting proapoptotic molecules.^{45,46}

In insulin signaling, the activation of Raf-1/MAPK occurs via IRS-1 or Shc^{23,47} and takes a central position in mitogenic pathways of insulin and a variety of other growth factors. For example, overexpression of IRS-1 induced activation of MAPK,²⁵ and adenovirus-mediated overexpression of IRS-1 interacting domains abolished insulin-stimulated mitogenesis, without affecting metabolic effects

like glucose transport.¹⁶ In Kym-1 cells, the partial inhibition of IR tyrosine phosphorylation after TNF treatment was correlated with a nearly complete loss of insulin-induced c-Raf-1/MAPK activation. At the molecular level, this could be due to at least two, nonexclusive mechanisms: First, it is apparent that a reduced IR kinase activity and correspondingly reduced IRS-1 activation should have effects on downstream substrates. Second, a direct interference of TNF signals with Raf-1 activation is conceivable. Indeed, evidence for the latter has recently been obtained by us in different cell models of a negative regulatory crosstalk of TNF with other typical mitogenic signals. Thus, we could show, e.g. for EGF-induced or v-src-induced MAPK activation, an inhibition of the MAPK cascade by TNF and its second messenger ceramide at the level of Raf-1.⁴⁸ Interestingly, ceramide, which is also reported to be an inhibitor of Akt/PKB,⁴⁹ has been shown to induce G0/G1 arrest, but not apoptosis, in cells lacking TNF receptors.⁵⁰ We here demonstrate for Kym-1 cells that, when the executing caspases are blocked, TNF is an effective cytostatic agent, able to completely inhibit cell proliferation of these transformed cells, apparently by interfering with several stages of the cell cycle.

Because of the multiplicity of signals conveyed by TNF, the growth arrest observed in Kym-1 cells is, unless caspases are blocked, a transient status, in which the cell is concomitantly primed for apoptosis. The rapid induction of cell death upon relief from caspase suppression after a 24 h TNF treatment in the presence of z-VAD-fmk is in accordance with the acquisition of a state of apoptotic dormancy, indicating that proapoptotic signals ensued in the presence of this caspase inhibitor to an irreversible commitment of the cells to die without further apoptotic signals by TNF. As discussed above, the interference of TNF with mitogenic signals simultaneously inhibits signal pathways leading to the activation of antiapoptotic molecules, such as IRS-1-dependent activation of Akt/PKB.⁵¹ The early inhibition of antiapoptotic survival signals by TNF observed here for Kym-1 cells is probably as important as generation of proapoptotic signals in defining cellular sensitivity to apoptosis. For Kym-1 cells the dominance of TNF over insulin action with respect to apoptosis induction is therefore not unexpected. However, for other cell models, a prevention of apoptotic signaling by insulin and FGF2 was demonstrated,^{20,45,52} indicating that in different cell types the extent and mode of signal crosstalk may differ significantly and thus largely influence the net cellular response to the same signals. The data together suggest that the cellular context determines whether the balance between pro- and antiapoptotic signals is shifted towards survival or rather cell death.

In conclusion, although the specific signal molecules within the TNF pathway responsible for interference with insulin signaling have yet to be revealed, it is apparent that TNF's crosstalk could be operative at multiple, receptor proximal and distal levels along mitogenic signal pathways, and thus makes it reasonable to assume that different TNF-induced signal molecules could be involved. In cells sensitive to TNF-induced apoptosis, such as Kym-1, the profound and lasting inhibition of mitogenic/anti-apoptotic

signals appears to be an early and likely important step towards progression to apoptosis.

Materials and Methods

Cell culture

The human rhabdomyosarcoma cell line Kym-1 was cultured in Click's RPMI 1640 medium supplemented with 10% FCS. Kym-1 cells were grown to approximately 80% confluence in tissue culture plates and, where indicated, were kept under low serum conditions (Click's RPMI 1640, 0.5% FCS) for 16 h before onset of experiments. Before treatment with TNF or insulin, Kym-1 cells were serum-deprived (Click's RPMI 1640, w/o FCS) for 2 h, and stimulated as indicated for various concentrations and times.

Cytokines, antibodies, and reagents

Recombinant huTNF was a kind gift of BASF, Germany. Insulin from bovine pancreas was purchased from Sigma. Antibodies specific for insulin receptor β (C19), IRS-1 (C20), STAT5 (G2, C17), c-Raf-1 (C12), PY (PY99), NF- κ B p65 (SC109), and NF- κ B p55 (SC114) were from Santa Cruz. 4G10 phosphotyrosine antibody was from Upstate. Secondary AP-linked antibodies, goat anti-mouse IgG and IgM (H+L) and goat anti-rabbit IgG (H+L), were from Dianova. STAT5 oligonucleotides (5'-AGATTCTAGGAATTCAATCC-3') were from Santa Cruz. NF- κ B oligonucleotides (5'-ATCAGG-GACTTTCCGCTGGGACTTTCCG-3') were synthesized by MWG. All inhibitors of proteases and phosphatases were from Biomol. Z-VAD-fmk (z-Val-Ala-DL-Asp-fluoromethylketone) was from BACHEM, HNMPA-[AM]₃ (Hydroxy-2-naphtalenylmethylphosphonic acid tris acetoxymethyl ester) was from Calbiochem, and Tyrphostin AG1024 was from ALEXIS Corporation. GST-[K71A] Erk1 agarose conjugate and non-active GST-MAP kinase kinase 1 (GST-MEK1) were from Upstate. Propidium iodide was from Sigma.

Cell lysis and immunoprecipitation

After stimulation the cells were washed twice with PBS (4°C) and scraped in 750 μ l ice-cold lysis buffer (50 mM Tris/HCl pH 7.4, 1% Triton X-100 (v/v), 150 mM NaCl, 5 mM EDTA pH 7.4, 1 mM NaF, 1 mM NaPP, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 100 nM okadaic acid, 100 nM calyculin A, 1 mM p-nitrophenyl-phosphate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 mM PMSF). After 1 h cell lysis, the lysates were centrifuged (10 000 \times g, 15 min, 4°C) and immunoprecipitation (1 μ g/ml antibody or antiserum per sample) was performed as described.¹² Protein contents in lysates were determined with the Bio-Rad Protein Assay using BSA as a standard.

Raf-1 kinase assay

After immunoprecipitation of c-Raf-1, the Protein-A pellets were washed three times (50 mM Tris/HCl pH 7.4, 150 mM NaCl), and resuspended in 30 μ l kinase buffer (20 mM Tris/HCl pH 7.4, 50 mM NaCl, 10 mM MgCl₂). After addition of 4 μ Ci [³²P]- γ -ATP (Amersham), and of substrates (500 ng GST-MEK1, 1 μ g GST-Erk1) in 10 μ l kinase buffer, the samples were incubated for 30 min at 37°C. The reaction was stopped by addition of 2 \times reducing Laemmli buffer. The samples were separated on 7.5% SDS-PAGE and transferred to nitrocellulose. Quantitative analyses of the kinase assays were performed with a phosphorimager (Molecular Dynamics).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were ³²P-labeled with polynucleotide kinase and EMSA was performed as described.¹² For 'supershift' assays nuclear extracts were incubated with the specific antibodies for NF- κ B subunits (10 μ l/ml) for 1 h at 4°C prior to the EMSA.

MTT assays and thymidine incorporation

For MTT proliferation assays, 10 000 or 2000 Kym-1 cells/well were seeded in 96-well plates in cell culture medium supplemented with 0.5% or 10% FCS. TNF, insulin, and/or inhibitors (z-VAD-fmk, Tyrphostin AG1024, HNMPA-[AM]₃) were added as indicated. After 48 h, the number of living cells was measured using the 'Cell Proliferation Kit I (MTT)' (Boehringer Mannheim), according to standard procedures. As a basal value, the MTT signal of an aliquot of cells seeded in parallel was determined directly after attachment (asterisks and dotted lines). For thymidine incorporation assays, 10 000 Kym-1 cells/well were seeded in 96-well plates in cell culture medium supplemented with 10% of FCS as indicated. TNF, insulin, and/or inhibitors (z-VAD-fmk, Tyrphostin AG1024, or HNMPA-[AM]₃) were added after attachment of the cells. Two hours after stimulation of the cells thymidine (1 μ Ci [methyl-³H] thymidine/sample) was added for 16 h. The cells were harvested in a cell harvester, and radioactivity incorporated into DNA was measured by scintillation counting according to standard procedures.

Flow cytometric determination of cellular DNA content

Cells were seeded in cell culture medium supplemented with 10% FCS. TNF, insulin and z-VAD-fmk were added as indicated, and incubated for 8 h. Detached and adherent cells were collected (30 min incubation with 0.02% EDTA), and washed twice in PBS containing 2% FCS (4°C). Cell number was adjusted to 2 Mio per ml. Three ml ice-cold ethanol was added to 1 ml cell suspension and the suspension was vortexed. The cells were stored overnight at 4°C, and were washed twice with PBS and stained for 3 h at 4°C with 1 ml PI-Solution (50 μ g/ml propidium iodide, 3.8 mM sodium citrate, 50 μ g/ml). The samples were analyzed in an EPICS XL-MCL (Coulter Immunotech Diagnostics).

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