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The somatostatin analogue TT-232 induces apoptosis in A431 cells Sustained activation of stress-activated kinases and inhibition of signalling to extracellular signal-regulated kinases

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Abstract

TT-232 is a somatostatin analogue containing a five-residue ring structure. The present report describes TT-232-induced signalling events in A431 cells, where a 4-h preincubation with the peptide irreversibly induced a cell death program, which involves DNA-laddering and the appearance of shrunken nuclei, but is unrelated to somatostatin signalling. Early intracellular signals of TT-232 include a transient two-fold activation of the extracellular signal-regulated kinase (ERK2) and a strong and sustained activation of the stress-activated protein kinases c-Jun NH_2-terminal kinase (JNK)/SAPK and p38MAPK. Blocking the signalling to ERK or p38MAPK activation had no effect on the TT-232-induced cell killing. At the commitment time for inducing cell death, TT-232 decreased EGFR-tyrosine phosphorylation and prevented epidermial growth factor (EGF)-induced events like cRaf-1 and ERK2 activation. Signalling to ERK activation by FCS, phorbol 12-myristate 13-acetate (PMA) and platelet-derived growth factor (PDGF) was similarly blocked. Our data suggest that TT-232 triggers an apoptotic type of cell death, concomitant with a strong activation of JNK and a blockade of cellular ERK2 activation pathways.

Keywords: Somatostatin-analog; Antitumour effect; Apoptosis; MAPKinases; A431 cells

1. Introduction

Somatostatin is a peptide hormone with pleiotropic biological effects, which are well documented and have recently been reviewed [1]. Its primary physiological functions include neurotransmission, inhibition of secretion by endocrine and exocrine cells, and the inhibition of cell growth of a variety of mammalian cancers, as well as the growth inhibition of a variety of transformed cell lines in culture. As an exception to this, somatostatin stimulates the proliferation of A431 cells [2]; its mechanism of action is largely unknown.

It has been suggested that somatostatin controls cell proliferation by activating intracellular signals, which negatively regulate mitogenic pathways [3–6]. In the human pancreatic cell line MIA PaCa-2, somatostatin has been reported to stimulate phosphotyrosine phosphatase activity in a pertussis toxin (PTX)-sensitive way, implicating a G protein dependent pathway [5,7,8]. Moreover, somatostatin was shown to stimulate the dephosphorylation of epidermal growth factor (EGF)[1]-receptor preparations in vitro [7,9].

Abbreviations: CHO cells; Chinese hamster ovary cells; DMEM; Dulbecco's modified Eagle's medium; EGF; epidermial growth factor; ERK; extracellular signal-regulated kinase; FCS; foetal calf serum; JNK; c-Jun NH_2-terminal kinase; MBP; myelin basic protein; PAGE; polyacrylamide gel electrophoresis; PBS; phosphate-buffered saline; PD98059; MEK inhibitor; PDGF; platelet-derived growth factor; PMA; phorbol 12-myristate 13-acetate; SB203580; p38MAP kinase inhibitor; PTX; pertussis toxin

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Transient expression of human somatostatin receptor 3 (SSTR3) in a v-Ras transformed NIH 3T3 cell line has also been shown to confer somatostatin-dependent inactivation of Raf-1 by the tyrosine dephosphorylation of the enzyme, presumably leading to the inhibition of extracellular signal regulated kinases (ERKs) [4]. However, somatostatin-mediated signalling through SSTR4 was reported to activate the ERK pathway [10,11]. Several somatostatin analogues have been developed as antiproliferative agents, such as sandostatin and RC-160 [12], but the exact mode of action of any of these compounds remains poorly understood and rather controversial.

Somatostatin analogues have been shown not only to inhibit mitogenic signalling pathways, but also to induce apoptosis in a multitude of cancer cell lines [13,14]. It is becoming increasingly clear that commitment of cells to programmed cell death or apoptosis, reflects a critical balance between the different MAPK-pathways leading to ERK, c-Jun NH_2-terminal kinase (JNK)/SAPK or p38 MAPK activation [15,16]. However, the involvement of the stress-activated kinases JNK/SAPK or p38 MAPK in somatostatin-induced antiproliferative or apoptotic events [17] has not been investigated.

We have recently developed a unique somatostatin analogue (TT-232) containing a five-residue ring structure: D-Phe-Cys*(S)-Tyr-D-Trp-Lys-Cys(S)*-Thr-NH_2. This analogue showed a strong in vivo and in vitro antiproliferative effect and induced apoptosis in several tumours or cancer cell lines, without inhibiting growth hormone release [14,18]. Because of its compact five-residue ring structure, TT-232 may be considered as a rather distant structural-analogue of the native somatostatin, which is reflected in the fact that the peptide does not exhibit any endocrine effects, and induces cell death only at much higher concentrations [14,18,19]. Except for the observation that TT-232 has the capacity to affect cellular tyrosine phosphorylation events [14,19,20], its mode of action is completely unknown. A recent report has suggested that TT-232 may mediate its effects through binding at low-affinity somatostatin receptors, and by its internalization to cytoplasmic and nuclear targets [21].

The present report shows that in the A431 cell line, TT-232 induces a robust and sustained activation of JNK/SAPK, concomitant with a commitment to programmed cell death. The activation of the JNK/SAPK pathway is paralleled by a general block in intracellular signalling to ERK-activation. Our data suggest that TT-232 triggers an apoptotic type of cell death program which is unrelated to somatostatin-receptor signalling events.

2. Experimental procedures

2.1. Materials

TT-232 was synthesized as described in Ref. [14]. Epidermal growth factor (EGF) was purchased from Boehringer Mannheim (Mannheim, Germany). Anti-EGFR, recombinant MEK1 (rMEK1) and recombinant ERK2 (rERK2) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-ERK2 antibody, anti-phospho-SAPK/JNK monoclonal antibody and anti-phospho-p38 MAP kinase antibody were from New England Biolabs (Beverly, MA, USA).

Antiphosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology (Lake Placid, CA, USA), and PVDF-blotting membranes were from Bio-Rad (Richmond, CA, USA). Protein A-TSK was from Affiland (Sart-Tilman, Belgium). [γ-(^{32}P)]ATP (10 mCi/ml) was from Amersham Life Sciences (Amersham, UK). The inhibitors, MEK inhibitor (PD98059) and p38MAP kinase inhibitor (SB203580), were from Alexis (San Diego, CA, USA); all other materials were from Sigma (St. Louis, MO, USA).

2.2. Preparation of peptide antibodies

Anti-ERK2 (amino acids 349–360), anti-JNK1 (amino acids 370–384), anti-c-Raf-1 (amino acids 638–648) antibodies were raised in rabbits against the COOH-terminal peptide sequences of the respective human proteins with the addition of an amino-terminal cysteine residue to facilitate linking of the peptides to KLH, BSA or agarose beads (Sulfolink coupling: Pierce, USA). The antibodies were affinity-purified on columns of their respective peptides linked to agarose.

2.3. Cell culture and preparation of extracts

A431 cells (ATCC CRL 1555) and A431 cells over-expressing platelet-derived growth factor (PDGF) β receptor [22] were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (containing 4.5 g/l of glucose) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin. Before the experiments, approximately 80% confluent A431 cells were starved for 48 h in serum-free DMEM. After a preincubation with TT-232 (1–30 μM dissolved in H_2O) for the indicated time periods, cells were washed once with ice-cold phosphate-buffered saline (PBS) and then lysed in buffer containing 25 mM Hepes (pH 7.5), 1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM Na_3VO_4 and 200 nM microcystin. After scraping and two times vortexing for 15 s, cell lysates were left on ice for 15 min and centrifuged at 15,000 $\times$ *g* for 15 min. The supernatants were either used immediately or stored at -80°C.

2.4. Colorimetric MTT (tetrazolium) assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in PBS at 5 mg/ml. One hundred-microliter stock solution of MTT was added per

milliliter medium in 24-well plates. Plates were incubated at 37°C for 4 h. Acidic isopropanol (0.04 N HCl in isopropanol) was added to all wells and mixed to dissolve the dark blue crystals. Then the plates were read on a Micro-Elisa reader, using the wavelength of 570 nm [23].

2.5. Hoechst staining for apoptotic cell death

Subconfluent A431 cells were grown on Lab-Tek Chamber slides and treated as described before. Then the cells were fixed in 100% cold methanol for 20 min, washed twice with cold PBS and stained for 15 min with 3 μM Hoechst 33342 dye (Sigma). After discarding the excess of the dye, cells were washed once with distilled water and covered by a drop of Vectashield (Vector Laboratories, Burligame, CA, USA). Fluorescent nuclei were visualized in fluorescence microscope (Nikon). Under these conditions, nuclei from living, apoptotic and necrotic cells could be clearly distinguished. Highly fluorescent, condensed nuclei that showed patches of compact chromatin were considered as being apoptotic.

2.6. Assessment of cell viability and morphology

Cells were seeded in Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL, USA) at 8×10^4 cells per chamber (22×22 mm) and incubated for 48 h at 37°C. The supernatant was then replaced by serum-free medium. Serum starved cells were treated with the indicated concentration of TT-232 for different periods of time. Cell viability was estimated by trypan blue exclusion [24]. Dead cells, which take up trypan blue, were counted and expressed as percentage of the total number of cells (a minimum of 500 cells were counted each time).

2.7. Cell cycle analysis (analysis of apoptosis by propidium iodide staining)

T75 flasks of A431 cells were grown to approximately 60% confluency and then treated with TT-232, after which cells were trypsinized and centrifuged ($300 \times g$ for 1 min). To the resultant pellet of 10^6 cells, 200 μl cold PBS and 1 ml of 70% ethanol at -20°C were added and incubated overnight at 4°C. The ethanol-treated cells were vortexed and centrifuged ($300 \times g$ for 1 min). The pellet was washed twice in PBS, incubated with 1 ml of 1 mg/ml RNAse (Sigma) and 100 μg/ml propidium iodide (Sigma) for 1 h at room temperature. Fluorescence of individual nuclei was monitored with a FACSort flow fluorocytometer (Becton Dickinson) using a 488-nm emission wavelength and a 650-nm excitation filter. Fifty thousand cells were analysed for each sample. Statistical analysis of the percentage of cells in each phase of the cell cycle was performed by the software provided with the instrument (Cell Quest software).

2.8. ERK-immunoprecipitation kinase assay

Cell lysates containing 150 μg of protein were incubated for 3 h with 3 μg of anti-ERK2 antibodies. Immunocomplexes were captured with 15 μl of protein A-TSK gel for 1 h. ERK2 immunoprecipitates were washed three times with PBS containing 1% NP-40, and twice with kinase assay buffer containing 20 mM MOPS (pH 7.6), 2 mM EGTA, 20 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4 and 0.1% Triton X-100. The washed beads were resuspended in 30 μl of kinase reaction mix containing the kinase buffer and 40 μM ATP, 5 μCi of [γ-^{32}P]ATP and 50 μg myelin basic protein (MBP) as kinase substrate. After incubation at 30°C for 15 min, the reaction was terminated by adding 5 vols. of SDS sample buffer and heating at 100°C for 3 min. The proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) using 11% gels, and the phosphorylated MBP protein was detected by autoradiography. Alternatively, the activation of ERK was also detected on Western blots, developed with antibodies that specifically recognize the dually phosphorylated (activated) enzyme.

2.9. JNK-immunoprecipitation kinase assay

Cell lysates containing 300 μg of protein were incubated for 3 h at 4°C with 5 μg of anti-JNK1 antibodies. The JNK-immunoprecipitation kinase assay was carried out essentially as described above for ERK2 except that 3 μg GST-c-Jun [25] was used as substrate, and the incubation time was 20 min. An alternative way to detect JNK-activation is its recognition by phospho-epitope specific JNK-antibodies on Western blots, as also mentioned for ERK and p38MAPK activations.

2.10. Detection of activated p38MAPK

Activation of p38MAPK was followed on Western blots, developed with antibodies that specifically recognize the dual phosphorylated (activated) enzyme.

2.11. c-Raf-1-immunoprecipitation kinase assay

Cell lysates containing 800 μg of protein were incubated for 3 h with 3 μg of anti-c-Raf-1 antibodies and processed as described for ERK2. The Raf-1 kinase activity was measured in a coupled kinase assay system combining recombinant MEK1, recombinant ERK2 and MBP: immune complexes were incubated for 15 min at 30°C with 125 ng rMEK1 and 300 ng rERK2 in 25 μl of a nonradioactive ATP-Mg mixture containing 20 mM $MgCl_2$ and 50 μM ATP. Finally, 15 μl of a mixture of 6 μCi [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 50 μg MBP was added and incubated for an additional 15 min at 30°C. The phosphorylated MBP protein was detected by autoradiography as outlined for the ERK2 assay.

2.12. Western blot analysis

The protein samples were subjected to SDS-PAGE, transferred onto PVDF membranes, blocked with 5% nonfat dry milk in Tris-buffered-saline containing 0.05% Tween 20, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized by ECL (Amersham-Pharmacia Biotech).

2.13. Determination of proteins

Protein concentrations were determined using the BCA Protein Assay kit (Pierce) according to the manufacturer's instructions using bovine serum albumin (BSA) as standard.

3. Results

3.1. TT-232 causes cell death in A431 cells

The in vitro antiproliferative effect of TT-232 has already been investigated using several tumour cell lines from different tissue origins [14]. The present report examines the effect of TT-232 on A431 human epidermoid carcinoma cells. Concentrations of TT-232 up to 1 μM had no effect, but significant cell killing was observed when incubating cells for 10 h with 15–60 μM of TT-232, as shown by the percentage of Hoechst positive cells, indicating that the cells died by apoptosis rather than necrosis (Fig. 1). This is also the effective concentration range reported for other cell lines [26]. The mechanism of TT-232-induced cell killing was further investigated using the near optimal dose of 30 μM.

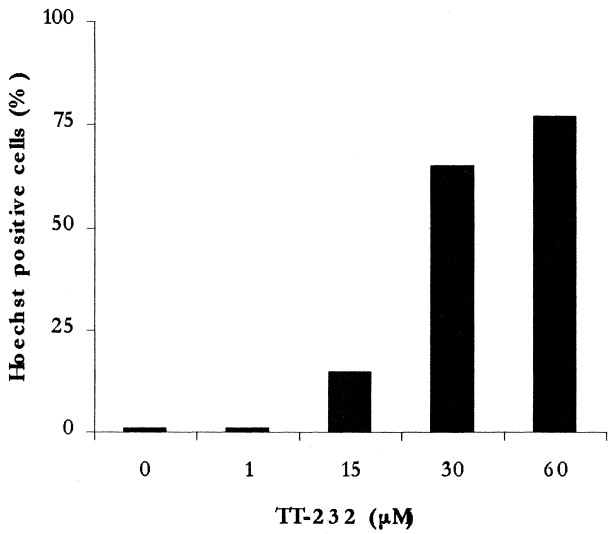

Fig. 1. Dose dependency of TT-232 induced cell death. Serum starved A431 cells were treated with different concentrations of TT-232 for 10 h. The extent of cell death was evaluated by Hoechst staining, counting the percentage of Hoechst positive cells.

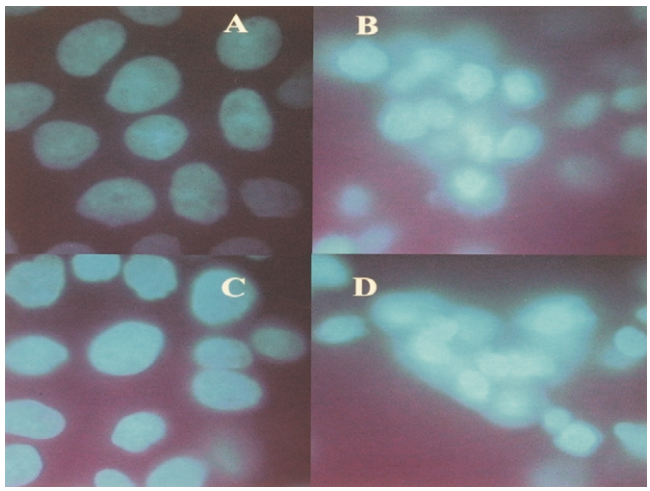

Fig. 2. (A–D) The TT-232 induced apoptosis in A431 cells is not effected by somatostatin signalling events. Serum starved A431 cells were left untreated or exposed to 30 μM TT-232 and/or 30 μM somatostatin for 24 h. After methanol fixation, cells were incubated with 3 μM Hoechst 33258 dye. Nuclear staining was examined with a fluorescence microscope. (A) Untreated (control) A431 cells, (B) cells treated with TT-232, (C) cells treated with somatostatin and (D) TT-232 and somatostatin cotreatment.

3.2. TT-232 induces apoptosis in A431 cells

Incubation of 48-h serum starved A431 cells with 30 μM TT-232 for 24 h in serum free DMEM caused substantial apoptosis, as shown by Hoechst staining of the cells (Fig. 2A,B). Substantial cell death, and formation of shrunken nuclei, was already visible under the light microscope after 6 h of treatment of the cells with TT-232 (not shown). In order to determine the time point where cells are committed to the programmed cell death, we preincubated A431 cells with 30 μM TT-232 from 1 to 6 h, after which the TT-232-containing medium was removed, the cells extensively washed, and further incubated in fresh DMEM. Cell survival was estimated after a total incubation time of 24 h evaluated by trypan blue exclusion and MTT assays (Table 1). There was no commitment to cell death after a 1-h incubation with the

Table 1
Cell survival after TT-232 treatment

Treatment	Living cells (% of total cell population)
Control	100
1 h* + 23 h	100
2 h* + 22 h	80
3 h* + 21 h	60
4 h* + 20 h	20
6 h* + 18 h	0
24 h*	0

48-h serum starved A431 cells were incubated with 30 μM TT-232 for the indicated times, then cells were washed, and incubated with serum-free DMEM in the absence of the peptide. Cell survival was evaluated at the 24-h time point.

* Hours of treatment with TT-232.

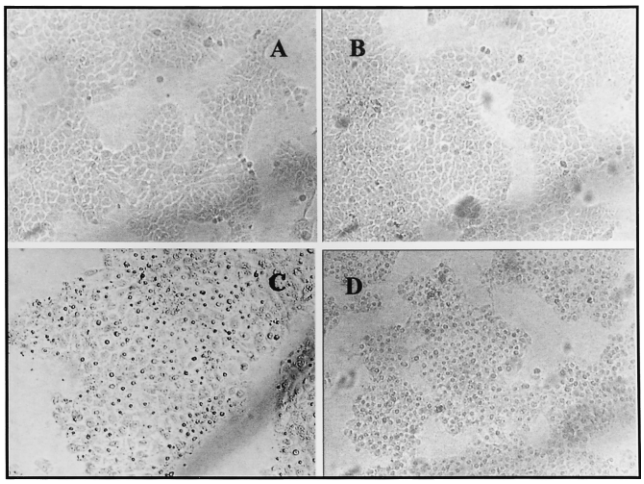

Fig. 3. (A–D) Light microscopic evaluation of TT-232 treated A431 cells. A431 cells were incubated with 30 μM TT-232 for the indicated times; then after a total 24 h of incubation, we used phase contrast light microscope to visualize the peptide-induced cell killing. (A) Control A431 cells, (B) 1 h of TT-232 treatment, evaluated after another 23 h, (C) 4 h of TT-232 treatment, evaluated after another 20 h and (D) 24 h TT-232 treatment.

peptide, and pretreatment of the cells with TT-232 for 2 or 3 h resulted in only partial cell killing over the 24-h time period. However, a 4-h preincubation resulted in a 70–100% cell death within 24 h (Fig. 3A–D). Similar results were obtained, when the induction of apoptosis was followed by FACS analysis (Fig. 4).

These results suggest that the very early intracellular signals which are elicited by the somatostatin analogue TT-232, are not sufficient for the commitment to the massive cell death seen at the later time points.

Treatment of A431 cells with TT-232 for more than 8 h, produced extensive ladder type of DNA fragmentation, confirming the results of the Hoechst staining as that cells died by apoptosis (Fig. 5).

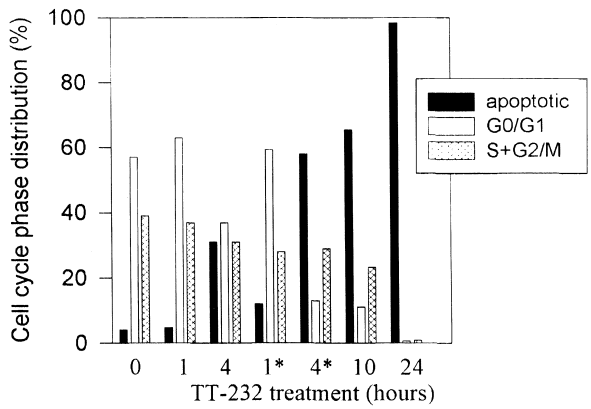

Fig. 4. Characterization of the TT-232-induced cell killing. A431 cells were trypsinized and ethanol-fixed as described under Section 2, and analysed by flow cytometry. The TT-232 commitment time for cell killing was determined by measuring the subG1 (apoptotic) fractions.

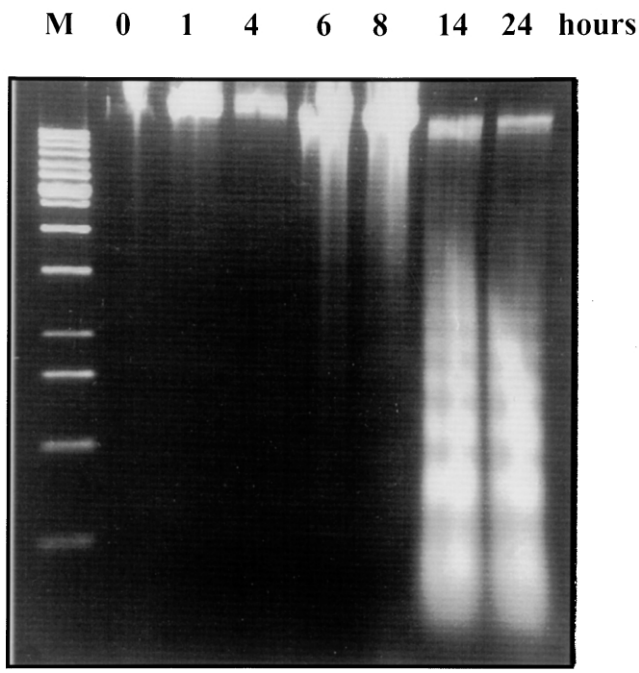

Fig. 5. Time course of TT-232-induced DNA fragmentation in A431 cells. Serum starved A431 cells were plated into T75 culture flasks and cultured to about 80% confluency. The cells were then exposed to 30 μM TT-232 in serum-free DMEM for the indicated times. Genomic DNA was isolated and run on 1.5% agarose gels. The DNA was visualized by ethidium bromide staining.

3.3. The TT-232-induced cytotoxic pathway is not affected by somatostatin 14-induced signalling events

The native somatostatin hormone (SST 14) was found to slightly promote cell proliferation in A431 cells (Fig. 6), as

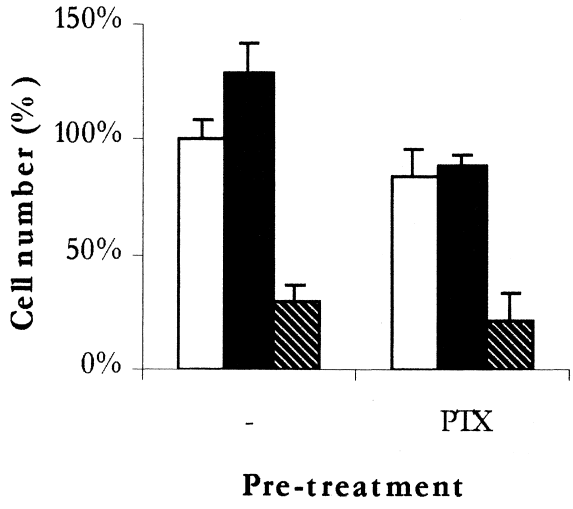

Fig. 6. Effect of PTX on TT-232 induced cell death. Serum starved A431 cells were left untreated or pretreated for 16 hours with 200 ng/ml PTX, and then exposed to either 10 μM somatostatin, or 30 μM TT-232 for another 24 h (untreated: empty bar, somatostatin solid bar, TT-232 striped bar). Cell numbers were evaluated by MTT test. Results shown here are representative of three independent experiments.

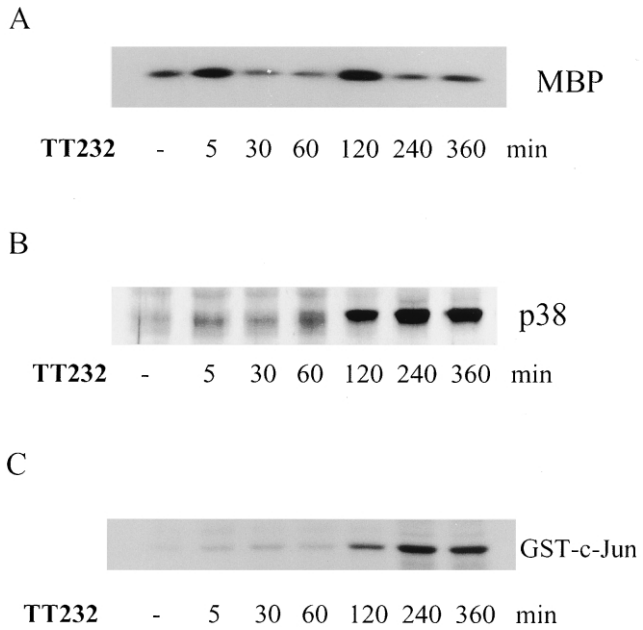

Fig. 7. Effect of TT-232 on the activation of ERK2, p38MAPK and JNK1 in A431 cells. Cells were incubated with 30 μM TT-232 for the indicated times. ERK2 (A) and JNK1 (C) activities were measured using MBP or GST-c-Jun as substrates, respectively, as described under Section 2. Results shown here are representative of at least three independent experiments. (B) The activation of p38MAPK was followed by the appearance of the dual phosporylated form of the enzyme on Western blots, visualized using phospho-specific p38MAPK antibodies.

also reported before [2]. Moreover, whereas SST 14 mediated signalling to cell proliferation was inhibited by pretreatment of cells with PTX, the TT-232 induced cytotoxicity was not affected (Fig. 6), suggesting that the cell killing was not mediated by G-protein signalling events.

We also investigated the possible interference of SST 14 with the TT-232 induced cell killing. Using a similar 30-μM dose of somatostatin, either as a pretreatment or as a cotreatment with TT-232, we found that somatostatin did not have any influence on TT-232-induced cell death in A431 cells (Fig. 2A–D). Furthermore, Chinese hamster ovary (CHO) cells, which do not express endogenous somatostatin receptors, were committed to cell death by TT-232 treament within the same 4 h of preincubation (not shown). This suggests that TT-232 may only be a distant structural analogue of SST 14 and that TT-232-induced cell killing is unrelated to somatostatin signalling events.

3.4. Time course of induction of ERK, p38MAPK and JNK/SAPK pathways by TT-232 in A431 cells

Serum starved A431 cells were incubated with 30 μM TT-232 for periods up to 6 h, and the induction of the different MAPK pathways was investigated at various times (Fig. 7A–C). In contrast to the relatively minor (two- to three-fold) and transient activations of ERK2 seen after 5-min and 2-h treatment of the cells with TT-232, a robust and sustained p38 MAPK and JNK1 activation was observed at the 2-, 4- and 6-h time points. The time course of activation of these stress-activated enzymes (Fig. 7B,C) parallels well with the time necessary for commitment to cell death (Table 1).

The importance of the ERK- and the p38MAPK-activation elicited by TT-232 was investigated by preincubation of the cells with TT-232 in the presence of the MEK-inhibitor PD98059, which selectively blocks signalling to ERKs, or the SB203580 compound, which selectively inhibits p38MAPK. Neither one of the two compounds could prevent the TT-232 induced cell death, whereas the inhibitors blocked their respective MAPK-pathway (not shown).

These results would suggest that the activation of the ERK and p38MAPK pathways is not necessary for the TT-232-induced cell death. It should be mentioned that PD98059 and SB203580 by themselves did not cause cell death in A431 cells at the concentrations used (not shown).

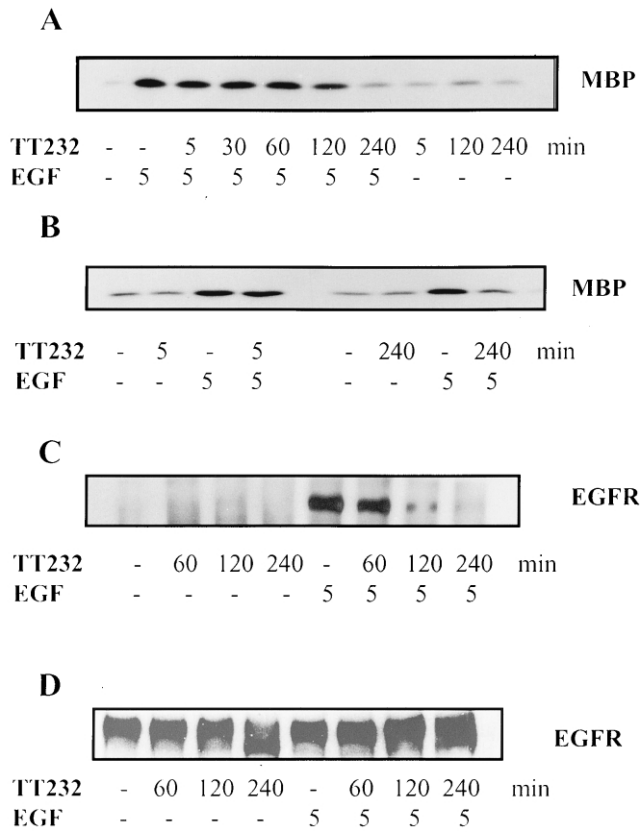

Fig. 8. Effect of 30 μM TT-232 on the EGF-induced activation of ERK2, c-Raf-1 and tyrosine auto-phosphorylation of EGFR in A431 cells. Cells were preincubated for the indicated times with TT-232, with or without a subsequent treatment with 100 ng/ml EGF for 5 min. ERK2 (A) and c-Raf-1 (B) activities were measured as described under Section 2. Results are representative of at least three independent experiments. (C) The tyrosine phosphorylation of the EGFR was evaluated by the reaction with antiphosphotyrosine antibodies on Western blots. (D) The stable expression of undegraded EGFR was visualized with anti-EGFR antibodies.

3.5. Pretreatment of A431 cells with TT-232 blocks subsequent EGF-mediated signalling

A431 cells express an unusually high level of EGFRs, and EGFR-mediated signalling to ERKs has been shown to be impaired by UVB-induced stress signals in various cell lines [25]. We therefore examined EGFR-mediated signalling in A431 cells, pretreated with TT-232 for different periods of time. ERK2-activation (Fig. 8A), c-Raf-1 activation (Fig. 8B) and tyrosine phosphorylation of the EGFR (Fig. 8C) were taken as readouts for EGFR-function.

In untreated A431 cells, maximal stimulations by 100 ng/ml of EGF was seen at the 5-min: a 10- to 15-fold activation of ERK2 and a three-fold activation of c-Raf-1 were generally observed (Fig. 6A,B). As a comparison, relatively minor TT-232-induced ERK activation was observed in the same experiment, as illustrated in the last three lanes of Fig. 8A. Pretreatment of cells with 30 μM TT-232 did not influence subsequent EGF-induced ERK2-activation at the early time points, but drastically blocked this event after the 3- to 4-h preincubation (Fig. 8A). The decrease in ERK-activity seen at these later time points did not result from degradation of the enzyme, as Western blots developed with anti-ERK2 antibodies showed the continuous presence intact enzyme. Similar results were obtained when c-Raf-1 activities were used as readout for EGF-induced signals (data not shown).

The tyrosine phosphorylation of the EGFR was investigated by Western blotting, where the receptor was visualized with anti-phosphotyrosine antibodies. A reproducible decrease in the phosphotyrosine content of the EGFR was already visible after 2 h of preincubation of A431 cells with TT-232, and the dephosphorylation was extensive at the 4-h time point (Fig. 8C). This coincides rather well with the time that cells were irreversibly committed to programmed cell death (Table 1). Blots developed with anti-EGFR antibodies showed the continuous presence of undegraded receptor (Fig. 8D).

Interestingly, signalling to the ERK-pathway by phorbol 12-myristate 13-acetate (PMA; 1 μM, 10 min), or FCS (10%, 10 min) (Fig. 9A), was also blocked after 4-h preincubation of the cells with TT-232. This may indicate that the interference of TT-232 with growth factor signalling to ERK is a general phenomenon, which may be linked to its cell killing mechanism. We therefore looked at the effect of TT-232 on the PDGF (30 ng/ml, 5 min)-mediated ERK activation, which can be taken as a paradigm of single growth factor signalling to ERKs. Since A431 cells do not express endogenous PDGF receptors, we used A431 cells stably transfected with the wild type PDGFR as described in Ref. [22]. As shown in Fig. 9B, TT-232 efficiently blocked PDGF-mediated ERK activation. The TT-232 induced cell killing was identical in parent A431 and in the PDGFR-expressing cells (not shown).

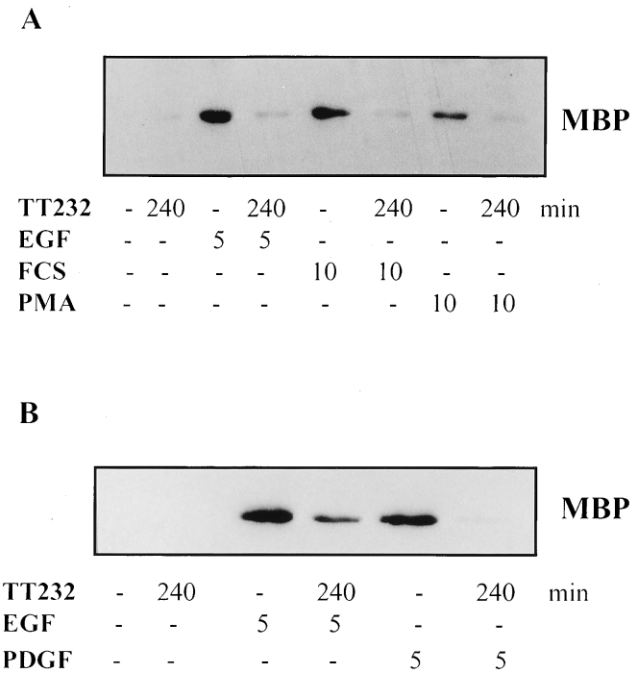

Fig. 9. Prolonged treatment with TT-232 blocks ERK2 activation in A431 cells in response to different stimuli. (A) A431 cells were stimulated with 100 ng/ml EGF for 5 min, 10% FCS for 10 min, 1 μM PMA for 10 min, after a 4-h, or without a TT-232 pretreatment. (B) Alternatively, PDGFR expressing A431 cells were treated with 100 ng/ml EGF for 5 min or with 40 ng/ml PDGF for 5 min in, again after a 4-h, or without a TT-232 pretreatment. The ERK activation was followed by the phosphorylation of MBP. The results are representative of three independent experiments.

4. Discussion

The present report describes intracellular signalling events elicited by TT-232 in A431 cells, where the cyclic peptide induces apoptosis in a time, and concentration, dependent way. The commitment time for cells to enter the apoptotic process was about 4 h at 37°C, so we do not expect the very early signals of TT-232 to be sufficient for the initiation of the programmed cell death. Several criteria, including the formation of shrunken nuclei (Hoechst staining), FACS analysis and DNA-laddering, showed that the TT-232-induced cell death was an apoptotic event.

TT-232, a unique somatostatin analogue without endocrine effects, is in the second phase of clinical trials after its antitumour activity was shown in a series of in vitro and in vivo experiments (4913/40/2000-TT-002-2001/OGYI, Hungary). It is only a distant structural analogue of somatostatin, and several lines of evidence suggest that TT-232 does not cause its antiproliferative and apoptotosis inducing effects through somatostatin receptors. First, TT-232 has been found to be equally effective in more than 50 tumour cell lines (NCI anticancer drug screening panel of cells), which do not all express the same extent of somatostatin receptors. Our results, moreover, indicate that CHO cells, which do not express somatostatin receptors at all, are just

as sensitive to TT-232-induced cell death as A431 cells. Second, somatostatin was shown to stimulate the growth of A431 cells, confirming an earlier report [2], and the native hormone did not affect the TT-232-induced cell killing in this cell line. Third, whereas somatostatin signals through serpentine receptors, which are sensitive to the inhibitory action of PTX, the TT-232-induced cell death or JNK1-activation was not.

Since the cyclic TT-232 is highly hydrophobic, it has the structural requirements to penetrate the cellular plasma membrane and could possibly be taken up by the cell by endocytosis. A rather slow uptake of the peptide by the cell would explain the long (4 h) preincubation, which is needed for the commitment to cell death. It is therefore likely that in order to produce its cytotoxic effect, TT-232 has to enter the cell.

Full characterization of the mechanism of action of the TT-232-induced cell death has been hampered by the fact that we have not been able to label the peptide with a fluorescent probe, while retaining its cytotoxic activity. There has been one report on the localization of tritium-labeled TT-232 on the cell surface, on cytoplasmic membranes and also in the nucleus of HT-29 human colon tumour cells [21]. The authors took this as suggestive evidence for the existence of low-affinity cell surface receptors for the peptide, while also implicating internalization of TT-232 in its apoptosis-inducing effect.

Interestingly, pretreatment of A431 cells with PTX, or with the MEK-inhibitor PD98059, completely abolished the early signalling of TT-232 to ERK-activation, while these treatments did not affect the commitment time for cell killing by the peptide. This is in concert with the data showing that a 1-h peptide treatment was not sufficient to induce cell death. We were also able to block the TT-232-mediated p38 MAPK activity with the SB203580 inhibitor, without affecting the peptide's apoptotic effect. This suggests that the ERK and p38MAPK activations are not necessary for the execution of the programmed cell death.

Exposure of cells to TT-232 activates the JNK pathway, as shown by the phosphorylation of the JNK enzymes, and by their activation measured by the increased phosphorylation of their substrate GST-c-Jun. The first appearance of stimulation of the JNK pathway precedes the TT-232 induced apoptotic process. Since there are currently no specific inhibitors of the JNK-pathway available, it is presently not possible to functionally link these pathways with the induction of apoptosis. An important phenomenon may be the inhibition of the EGF-induced signalling to ERK activation, which is mostly evident around the time that TT-232 causes the commitment for A431 cell death. The blocking of the ERK-cascade of events is a general phenomenon, and not restricted to EGF-signalling, since TT-232 pretreatment of cells for 4 h also greatly impaired signalling to ERK-activation by FCS, PMA and PDGF. An elevated ratio of JNK to ERK activity has been proposed as a proapoptotic condition [27,28], and an irreversible inhibition of the ERK-pathway has been reported for other stress signals that induce apoptosis, such as UVB-irradiation [25].

In conclusion, our data suggest that TT-232 triggers an apoptotic type of cell death, concomitant with a strong activation of JNK and a blockade of cellular ERK2 activation pathways.

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