

Induction of Apoptosis in Hematopoietic Cells with an Antibody Against Tomoregulin-1

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Abstract. *Tomoregulin-1, a type-I transmembrane protein with two follistatin modules, a unique epidermal growth factor (EGF) domain and a short, highly conserved cytoplasmic tail, was studied. A number of hematopoietic cell lines (L1210, CEM, Jurkat, U937, K562, JY, THP-1 and T2) express tomoregulin-1 endogenously. In these cells, apoptosis was induced by an antiserum (C29) and purified IgG against the follistatin modules, but not by antisera against the EGF-domain or the cytoplasmic tail. Furthermore, C29 induced apoptosis in tomoregulin-1-, but not in mock-transfected cells. Apoptosis was monitored through genomic DNA fragmentation, annexin-V staining and caspase-3 activation. Treatment of the cells with C29 in the presence of H89 (a Ser/Thr kinase inhibitor) or 8'-bromo-cyclicAMP revealed that apoptosis was mediated by a cAMP-dependent Ser/Thr kinase. Moreover, C29 increased [cAMP]_i over 5-fold. Together, these data suggest that the C29 antiserum against tomoregulin-1 induces apoptosis of hematopoietic cells.*

During the application of a differential screening approach to *Xenopus laevis* pituitary, several novel cDNAs have been discovered (1). One of these cDNAs was designated 7365 and encodes a protein structurally related to the transmembrane precursors of the epidermal/transforming growth factor (EGF/TGF- α) family with a unique EGF domain, in that all characteristics are present except for an essential Arg residue (2). In addition, the 7365 protein

contains a signal peptide, 2 extracellular follistatin (FS) domains, a transmembrane domain and a short cytoplasmic C-terminal region. Two papers described expression patterns of 7365 (3, 4). The mouse and human orthologues (GenBank, U19878) were cloned, and the name tomoregulin-1 was proposed for this protein (3). A striking degree of amino acid sequence identity between mammalian and amphibian tomoregulin-1 was found, in particular in the 29 amino acid long C-terminal portion of the protein (4). The short cytoplasmic tail is nearly identical (97%) in mammals and *Xenopus laevis*. In view of the above characteristics, we decided to investigate the role of tomoregulin-1 in the proliferation and apoptosis of mammalian cell lines. For this study, antisera directed against different parts of tomoregulin-1 were employed. The use of antisera to unravel signaling pathways has been successful for CD95 and the amyloid precursor protein (5, 6). Following the two reports of expression patterns (3, 4), evidence that tomoregulin-1 is involved in Ser/Thr kinase-dependent apoptosis of hematopoietic cells is provided here.

Materials and Methods

Cell lines. JY (human EBV-transformed B-cell) and T2 (human/mouse T-cell hybridoma) were cultured in Iscove's medium supplemented with 5% fetal calf serum (FCS). CEM, Jurkat (both human T-cells) and U937 (human histiocytoma) were cultured in 5% FCS supplemented DMEM. K562 (human erythroleukemic cell) and THP-1 (human monocytic cell) were grown in HEPES-buffered RPMI supplemented with 10% FCS and L1210 (mouse lymphocytic leukemic cell) in DMEM with 4.5 g glucose/L and 10% horse serum. All growth media contained antibiotics (penicillin/streptomycin) and the cell lines were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. L1210 was a gift from Dr. J. Kuiper (Leiden, The Netherlands). The other cell lines were kindly provided by Dr. C. Figdor (Nijmegen, The Netherlands).

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Chemicals. Tissue culture materials were obtained from BioWhittaker Europe (Verviers, Belgium) and Greiner (Alphen aan den Rijn, The Netherlands), H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) was supplied by Calbiochem (Breda, The Netherlands) and 8'-bromo-cyclicAMP was provided by Sigma (Zwijndrecht, The Netherlands).

Antisera. Antiserum B73 was raised against the 12 most C-terminal amino acids of human tomoregulin-1, with an N-terminal Cys to couple to hemocyanin, which was used as a carrier in the immunization. Antiserum A59, raised against the EGF-like-domain of human tomoregulin-1, has been described previously (2). Antiserum C29 was raised against a recombinant polypeptide corresponding to the follistatin domains of human tomoregulin-1 (amino acids 114-218) expressed in *E. coli* as a fusion protein with a hexahistidine tag (Qiagen, Hilden, Germany).

Cytotoxic assays. Cytotoxicity was analyzed by the MTT (adherent cells) or XTT (suspension cells) proliferation assay (Boehringer-Mannheim, Germany) and by means of manual counting on *ad random* fields in a buckler counting chamber.

Transfection. Transient transfection was performed with the Lipofectamine 2000 (Invitrogen, Groningen, the Netherlands) and full-length human tomoregulin-1 cDNA in the pCS2 vector under the regulation of a CMV promoter. Two days after transfection, C29 was added (10^3 -fold diluted), and two days later cell numbers were analyzed as described above. Transfection experiments were done twice with each in triplicate.

DNA fragmentation. Cells were lysed for 2 h at 50°C in a nuclear lysis buffer containing 50 mM Tris pH 8.0, 300 mM NaCl, 5 mM EDTA, 15 mM MgCl₂ and 1% NP40. DNA was precipitated with 2 volumes of ethanol, followed by 30 min incubation at -80°C, centrifugation (13000 rpm) for 30 min followed by analysis on a 1.7% agarose gel in the presence of EtBr.

Western blotting. Standard procedures were used. Cells were lysed with nuclear lysis buffer as under DNA fragmentation with the addition of protease inhibitors. The polyclonal rabbit antiserum against poly(ADP-ribose)polymerase (PARP) was kindly provided by Dr. M. VanderCraen (Ghent, Belgium); the polyclonal rabbit antiserum against p53 was kindly provided by Dr. G. Boonen (Utrecht, The Netherlands). Enhanced chemo luminescence (ECL) chemicals were derived from Amersham/Pharmacia Biotech (Roosendaal, The Netherlands).

Phosphatidylserine staining. To measure phosphatidylserine (PS) exposure on FACS, cells were labelled with annexin-V-FITC, which specifically binds PS (41).

Caspase-3 activity. The activity of caspase-3 was measured fluorometrically by the release of 7-amino-4-methylcoumarin (AMC) from a Acetyl-DEVD-7-AMC, as described previously (42).

[cAMP]_i. Following lysis of the cells with 3.5% HClO₄ and subsequent neutralisation with KHCO₃, samples were analyzed with a commercially available RIA (Amersham, Uppsala, Sweden) (43).

IgG purification. IgGs were isolated from the serum with a Sepharose-A column and elution with Tris-HCl (44).

Results

Structural and genetic information suggested that tomoregulin-1 could play a role in proliferation or apoptosis. For example, the presence of the EGF-like domain and the FS modules that can bind pro-apoptotic activin indicates a role for tomoregulin-1 in the apoptotic process (7-10). Its localization on human chromosome 9q31 (11) also represents a region associated with several neuropathies, loss of heterozygosity, deletions and the presence of tumor suppressors (12-25). Thus, the possibility that tomoregulin-1 is involved in apoptosis or proliferation was investigated.

It was first established, by RT-PCR analysis of tomoregulin-1 mRNA, that a number of cell lines of hematopoietic origin (L1210, CEM, Jurkat, U937, K562, JY, THP-1 and T2) express tomoregulin-1 endogenously. No expression was observed in the adherent carcinoma cell lines T24 (human bladder carcinoma), CaCo-2 and HT29 (both human colon carcinomas), CMT8 (canine mammary carcinoma) or PC12 (rat pheochromocytoma). All subsequent experiments were performed with the mouse lymphocytic leukemic cell line L1210. L1210 cells was treated with several antisera directed against different parts of tomoregulin-1, namely A59 (raised against the EGF-like domain), B73 (raised against the 12 most C-terminal amino acids of tomoregulin-1) and C29 (raised against a recombinant polypeptide corresponding to the FS domains of tomoregulin-1). As a control, no serum was added to the cells, or the cells were exposed to pre-immune serum, normal rabbit serum (NRS) or serum against secretogranin-III (a neuroendocrine secretory protein). None one of these conditions, nor the A59- or B73-treated cells, showed a reduction in cell number (Figure 1A). Only C29, the antiserum against the FS domains of tomoregulin-1, clearly reduced cell numbers in a concentration-dependent fashion. A 10^3 -fold dilution of C29 in growth medium reduced cell numbers to ~50% of the untreated L1210 cells. No effect of C29 was observed when the serum was diluted 10^5 -fold or more. The cytotoxic effect could be prevented by the addition of an excess of the recombinant follistatin domain of tomoregulin-1 or by pre-treating C29 with goat-anti-rabbit serum. The effect of 10^3 -fold diluted C29 was not restricted to L1210 cells, since similar results were obtained with CEM (64% survival), Jurkat (54% survival), U937 (45% survival), K562 (76% survival), JY (69% survival), THP-1 (73% survival) and T2 cells (74% survival). No effect of C29 was observed on cells lacking (no PCR product after 40 PCR-cycles) endogenous tomoregulin-1, such as T24, CaCo-2, HT29, CMT8 and PC12 cells (data not shown). The effect of C29 on the growth curves of L1210 cells is shown in Figure 1B. The growth curves of control cells and C29-treated cells, differing by about one doubling-time,

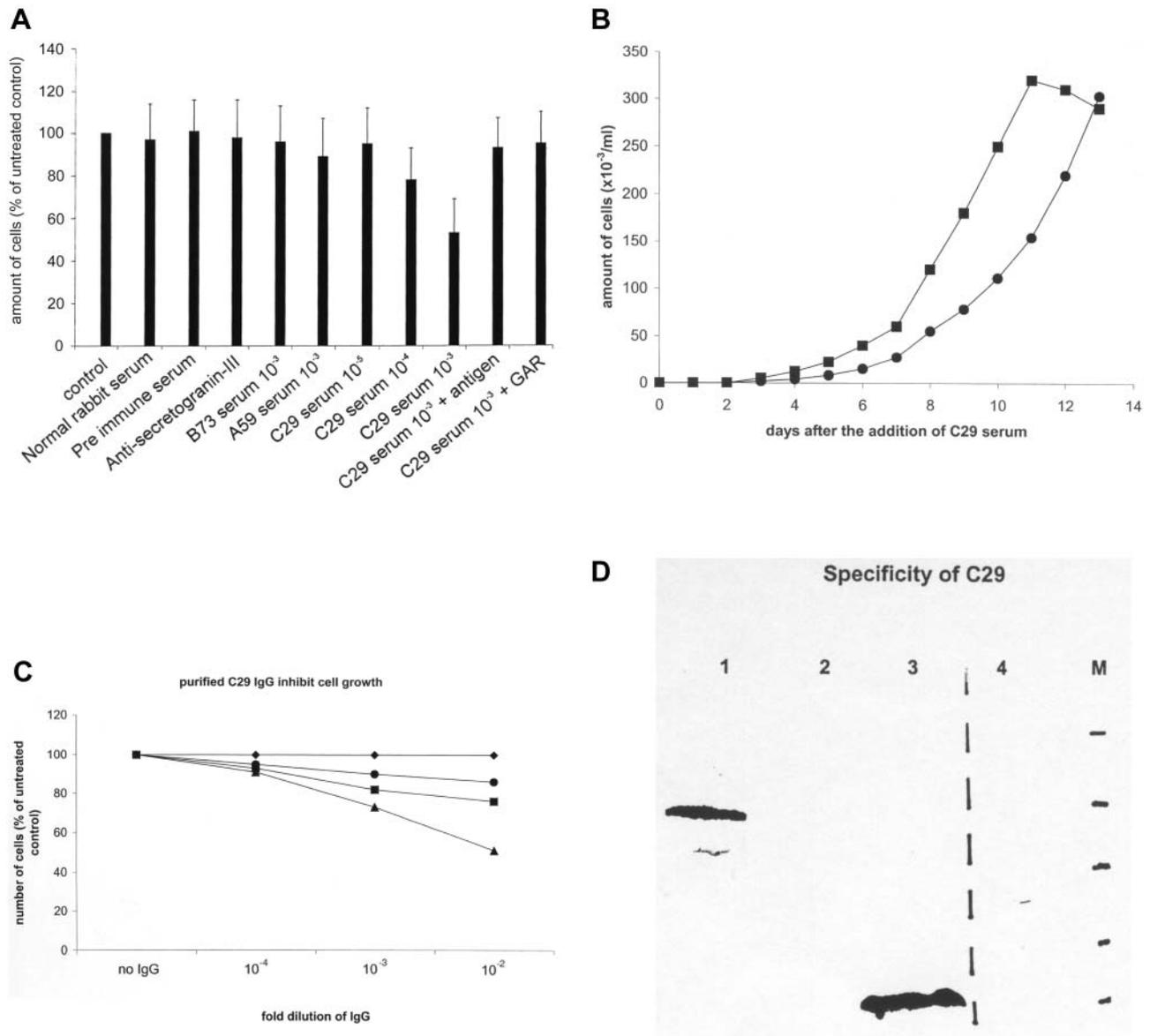


Figure 1. The effect of anti-tomoregulin-1 sera on the viability of L1210 mouse lymphocytic leukemic cells. (A) On day zero, cells were seeded in fresh medium supplemented with sera of different dilutions. On day 5, cells were counted as described in Materials and Methods. Antiserum B73 was raised against the 12 most C-terminal amino acids of human tomoregulin-1, with an N-terminal Cys to couple to hemocyanin, the antiserum A59, raised against the EGF-like-domain of human tomoregulin-1. Antiserum C29 was raised against a recombinant polypeptide corresponding to the follistatin domains of human tomoregulin-1 (amino acids 114-218) expressed in *E. coli* as a fusion protein with a hexahistidine tag. Goat-anti-rabbit (GAR) and the C29-antigen were pre-incubated with C29 1 h prior to the addition to the cells. Control cells were incubated in normal growth medium (without any further additions), and normal rabbit serum (NRS) and pre-immune serum were added 10³-fold dilutions. (B) At day zero, cells were seeded in fresh medium supplemented with 10³-fold dilution of C29. Squares indicate NRS treatment; closed circles represent C29 treatment. Each day, cell numbers were measured as described in Materials and Methods. (C) The effect of purified IgGs on cell viability. Fraction 2 (circles) and fraction 20 (diamonds) contain no protein and serve as controls for the elution buffer; fraction 4 (squares) and fraction 5 (triangles) contain the highest amounts of IgG. The experiments were repeated at least 3 times with 4 independent samples and the standard deviation was less than 10%. (D) Western blotting to measure the specificity of the C29 antiserum. Both the primary antibody C29 and the peroxidase-labelled goat-anti-rabbit antibody (the second antibody) were incubated for 3 h at room temperature. Lane 1, lysate of L1210 cells; lane 2, lysate of T24 human bladder carcinoma cells; lane 3, lysate of *E. coli* expressing C29 antigen; lane 4, lysate of L1210 cells, first antibody C29 pre-incubated with C29 antigen. Lane 4 was treated separately from lanes 1-3. Each lane contained a similar amount of protein. The sizes of the marker proteins are 66, 46, 35, 21.5 and 14.2 kDa.

A. Effects of different antisera on L1210 proliferation. B. Effect of C29 antiserum on L1210 proliferation. C. Effect of purified IgGs against the follistatin domain of tomoregulin-1 on L1210 cells. D. Western blot showing that the C29 antiserum recognizes only tomoregulin-1.

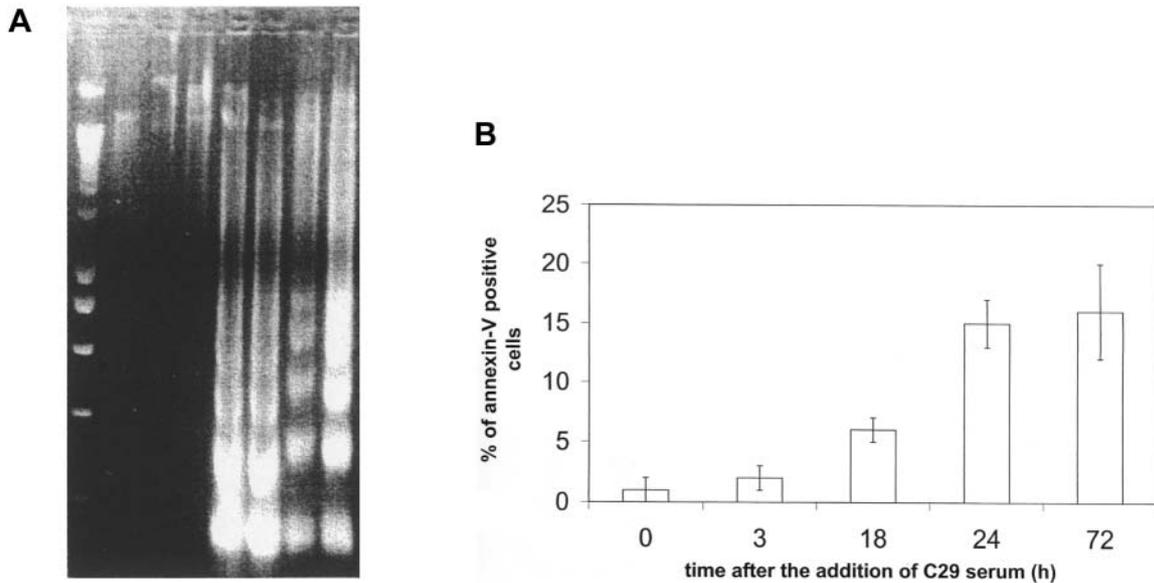


Figure 2. Anti-tomoregulin-1-FS serum (C29) induces apoptosis in L1210 cells. (A) C29 induces DNA fragmentation. Lane 1, 200 bp ladder; lane 2, NRS-treatment no C29; lane 3, C29 treatment for 1 h; lane 4, C29 treatment for 3 h; lane 5, C29 treatment for 6 h; lane 6, C29 treatment for 16 h; lane 7, C29 treatment for 24 h; lane 8, cycloheximide treatment for 24 h. (B) C29 increases the amount of annexin-V positive cells. Antisera were diluted 1000-fold. The experiment was performed 3 times; representative experiments are shown.

A. C29 antiserum induced DNA fragmentation in L1210 cells. B. C29 antiserum induced apoptosis, as measured with annexin-V, in L1210 cells.

indicate that C29 did not have an effect on the duration of the cell cycle, but rather induced cell killing. These data also confirm that in the concentrations used, C29 induced a cell death of ~50%. Indeed, a C29-mediated effect on the cell cycle distribution (control cells 51% in G1-, 41% in S- and 8% in G2/M-phase; C29-treated cells 48% in G1-, 38% in S- and 14% in G2M-phase) was not observed. These measurements were made two days after the addition of C29 and, therefore, the (apoptotic) subG1 cells are absent due to complete disintegration of these cells. Furthermore, the parallel growth curves indicated that the cytotoxic effect induced by C29 was initiated soon after the addition of the antiserum and subsequently disappeared, possibly due to a reduced efficacy of the serum. The plateau in the growth curves was caused by the fact that the medium was not refreshed during the course of this experiment. Whether the fact that the cells are sensitive to C29 only shortly after the addition of C29 is due to the breakdown of C29, reduced levels of tomoregulin-1 after C29 addition, the induction of anti-apoptotic factors or tolerance, remains to be investigated.

The effect of Sephadex-purified C29-IgG is shown in Figure 1C. The fractions with the highest IgG content clearly induced a reduction in cell number, whereas fractions without detectable amounts of IgG did not elicit such an effect. The data in this figure thus clearly indicate that the C29-mediated response was elicited by IgGs rather

than an unknown agent. Since C29 recognized a single band of the expected size (~41 kDa) in lysates from L1210 cells (Figure 1D), we can conclude that C29 is specific for tomoregulin-1. Moreover, following pre-incubation of the antibody with the recombinant antigen, the 41 kDa-band disappeared (Figure 1D, lane 4). Lysates of tomoregulin-1-negative T24 and Chinese hamster ovary cells gave no immuno-reactive product, and no product with a size similar to that of the structurally, related FS (26), was observed (Figure 1D, lane 2, and data not shown).

Since we cannot exclude that C29 recognizes an epitope on hematopoietic cells that is different from tomoregulin-1, tomoregulin-1-deficient CMT8 cells were transiently transfected with a construct encoding tomoregulin-1 and C29-induced growth inhibition was measured. Interestingly, the tomoregulin-1-transfected cells displayed a growth advantage compared to mock-transfected cells (~35% more cells). In mock-transfected cells, C29 had no effect on the growth curves (88±6 cells/volume without C29 and 83±7 cells/volume after C29 addition). However, the addition of C29 to cells transfected with tomoregulin-1 led to apoptosis (~60% less cells; 119±17 cells/volume without and 48±15 cells/volume with C29). These observations indicate that C29 specifically recognizes tomoregulin-1 and that tomoregulin-1 is a survival factor (e.g. a growth factor, a growth factor receptor or a decoy receptor for growth inhibitors).

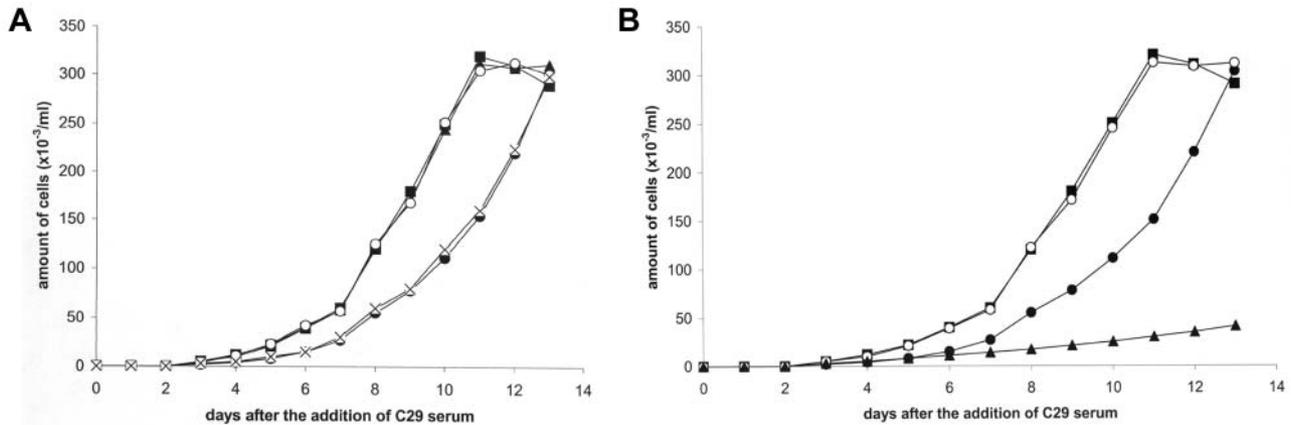


Figure 3. Ser/Thr kinases are involved in anti-tomoregulin-1-FS serum (C29)-mediated apoptosis. (A) The effect of H89, an inhibitor of cAMP-dependent Ser/Thr kinases on C29-induced apoptosis. Squares represent NRS treatment; closed circles represent C29 treatment; triangles represent C29 treatment plus 10 μM H89; open circles represent NRS treatment plus 10 μM H89; crosses represent C29 treatment plus 100 nM H89 (B) The effect of 8'-bromo-cAMP on C29-induced apoptosis. Squares represent NRS treatment; closed circles represent C29 treatment; open circles represent 100 μM 8'-bromo-cAMP; triangles represent C29 treatment plus 100 μM 8'-bromo-cAMP. To allow cellular uptake, drugs were added 1 h prior to the addition of C29. Cell numbers were measured as described in Materials and Methods and C29 was diluted 10^3 -fold. The experiments were repeated at least 3 times with 4 independent samples, and the standard deviation was less than 10%.

A. The Ser/Thr kinase inhibitor H89 protects L1210 cells against C29-induced apoptosis. B. The cAMP analogue 8'-bromo-cAMP strongly stimulates C29-induced apoptosis in L1210 cells.

Cytotoxicity is mediated either by an apoptotic or a necrotic pathway. In order to discriminate between these two fundamentally different processes, 4 assays were performed: DNA fragmentation, PARP proteolysis, annexin-V staining and caspase-3 activation. As shown in Figure 2A, C29 induced the fragmentation of genomic DNA isolated from L1210 cells. As early as 4 hours after the addition of the antiserum, the L1210 DNA was cut into oligosomal DNA fragments (lane 3 in Figure 2A), suggestive of the induction of apoptosis. Longer exposure to C29 resulted in even more DNA laddering, comparable to that of cycloheximide-treated L1210 cells (positive control in lane 7, Figure 2A). DNA ladder formation was not restricted to L1210 cells, because similar results were observed in K562 and THP-1 cells (data not shown). The second assay used to establish the process of apoptosis was the staining of cells with annexin-V, measuring the shift of phosphatidylserine (PS) from the inner leaflet of the plasma membrane towards the outer leaflet. A clear increase in the percentage of annexin-V-positive cells was observed (Figure 2B). Within 3 h of treatment the amount of apoptotic cells increased from 2.5% to 6%, whereas 24 h of C29 treatment resulted in a significant increase to ~15% annexin-V-positive cells. These cells remained negative for propidium iodide (PI) and, thus, no signs of necrosis were observed during this time period. However, 3 days after the addition of C29, annexin-V-positive cells also became positive for PI, implying that these cells had undergone secondary necrosis. Finally, the effect of C29 on caspase-3 activity was

measured. The activity increased from 5.8 ± 0.1 pmol AMC/min/mg for untreated cells to 7.4 ± 0.3 pmol AMC/min/mg 4 h after the addition of C29, while at 24 h caspase-3 activity doubled to 12.3 ± 1.2 pmol AMC/min/mg. Western blot analysis revealed a C29-induced cleavage of the 116-kDa poly(ADP-ribose)polymerase (PARP) into a 25-kDa and an 85-kDa fragment (27,28) and an increase in p53 after the addition of C29 (data not shown). Taken together, the results obtained from the different assays showed that C29 induces apoptosis in L1210 cells.

A region within tomoregulin-1 involved in the apoptotic process may be its cytoplasmic tail. This 29 amino acid-tail is virtually identical between mammals and *Xenopus laevis* (97% identity, 100% similarity) and is rich in Ser/Thr residues (6 out of 29, 5 of the C-terminal 9 residues). Therefore, we investigated the role of Ser/Thr kinases in C29-mediated apoptosis. The effect of the Ser/Thr kinase inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) on C29-induced apoptosis is shown in Figure 3A. The inhibition of cAMP-dependent Ser/Thr kinases clearly protected L1210 cells against the apoptotic effect of C29, whereas H89 alone had no effect on the L1210 cells. Protection was already observed with H89 concentrations as low as 0.5 μM , while complete protection was obtained at 10 μM H89. Protection did not occur when H89 was added 2 days after the addition of C29 (data not shown). This finding indicates that the cytotoxic effect induced by C29 is initiated soon after the addition of the antiserum and, thus, corroborates the conclusions derived from the growth curves in Figures 1B and the 4 apoptosis assays.

Because we cannot exclude the possibility that H89 had effects in addition to its well-known inhibition of cAMP-dependent Ser/Thr kinases, the effect of drugs that mimic cAMP-dependent processes were measured (Figure 3B). In combination with C29, a strong synergy was observed by 8'-bromo-cAMP (cell-permeable and stable analogue of cAMP). At a concentration of 100 μ M 8'-bromo-cAMP, almost complete suppression of viability was observed. The used concentrations of both H89 and 8'-bromo-cAMP were well within the range normally applied (29, 30). Thus, the effects of the inhibition (by H89) and the stimulation (by 8'-bromo-cAMP) of Ser/Thr kinases suggested a role for these kinases in C29-mediated apoptosis. To further substantiate these inhibition/stimulation studies, the effect of C29 on the intracellular cAMP levels was measured. As depicted in Figure 4, a rapid and substantial increase in [cAMP]_i was induced by C29. As soon as 1 h after the addition, a significant increase was observed (4-fold), whereas 24 h after the addition of C29 [cAMP]_i was even further increased, up to 10-fold.

Discussion

In this study, we used a number of antisera against different domains of tomoregulin-1 to dissect its function. Only the antiserum directed against an epitope within the follistatin domains induced apoptosis of L1210 cells. For a number of reasons, we conclude that the anti-tomoregulin-1 antibodies in the C29 serum are responsible for the observed effects. First, antisera raised against other parts of tomoregulin-1 (the extracellular EGF-like domain and intracellular cytoplasmic tail) as well as antibodies against the unrelated neuroendocrine secretory protein (secretogranin III) were not effective. Second, pre-immune serum had no effect on cell numbers, nor did normal rabbit serum. Third, pre-treatment of C29 with specific antibodies (goat-anti-rabbit) completely prevented C29-mediated apoptosis. Fourth, no effect was observed in cells lacking endogenous tomoregulin-1, such as T24, CaCo-2, HT29, CMT8 and PC12 cells. Fifth, excess of the C29 antigen fully abrogated C29-induced apoptosis. Finally, Sepharose-A purified C29-IgGs induced a concentration-dependent cell death. The specificity of C29 was shown by transient transfection studies revealing that in tomoregulin-1 but not in mock-transfected CMT8 cells, C29 induced apoptosis.

The induction of apoptosis by the C29 antiserum suggests that a modification of the activity of tomoregulin-1 is involved in an apoptotic pathway, but it is unclear whether this is caused by an activation or inactivation of tomoregulin-1. The possibility that C29 blocks tomoregulin-1 activity is more likely than that it increases its activity, based on functional studies with a close relative of tomoregulin-1, tomoregulin-2 (~50% amino acid sequence identity) (31). A human brain tomoregulin-2 cDNA was identified by PCR-analysis using degenerate primers corresponding to an EGF-consensus

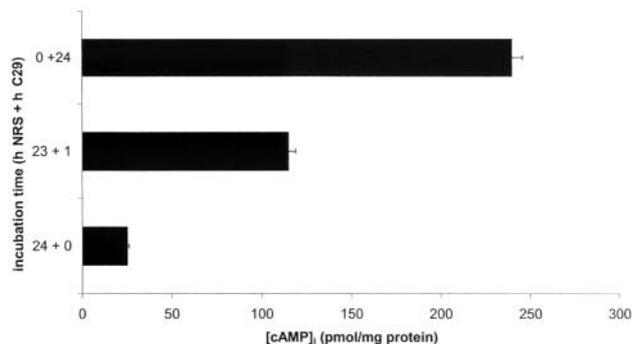


Figure 4. Anti-tomoregulin-1-FS serum (C29) increases the amount of intracellular cAMP. Lowest bar, control cells treated for 24 h with NRS; middle bar, cells treated for 24 h with NRS and the last hour with 10^3 -fold diluted C29; highest bar, cells treated for 24 h with 10^3 -fold diluted C29. Intracellular cAMP levels are induced by C29 treatment in L1210 cells.

sequence. Tomoregulin-2 was found to weakly tyrosine phosphorylate the erbB-4 receptor, suggesting that it may be involved in cell growth, differentiation or apoptosis (31). Furthermore, tomoregulin-2 has been found to act as a hippocampal and mesencephalic neuronal survival factor, and it may be involved in colorectal polyps and cancer, as well as prostate cancer progression (32-35). With respect to the latter, it is interesting to note that tomoregulin-2 is involved in androgen-independent growth of prostate cancer (34, 35). In addition, cleavage of tomoregulin-2 in the extracellular domain has been shown (31, 35, 36) and soluble forms of this protein have been found (31). In view of the structural resemblance between the tomoregulins, tomoregulin-1 may have a physiological function similar to that of tomoregulin-2, thus acting as a growth factor, a growth factor receptor or a decoy receptor for growth-inhibiting ligands. This is in line with our findings that tomoregulin-1 is highly expressed in lung tumors (Eib *et al.*, unpublished observations) and in choriocarcinomas *in vitro* (BeWo cell line) and *in vivo* (Penning *et al.*, unpublished observations). Moreover, during urodele limb regeneration and mouse embryonic limb development, tomoregulin-1 is up-regulated, suggestive of a role in growth factor signaling (4). Finally, 2 recent papers indicated an effect of tomoregulin on nodal signaling, again emphasizing a possible role of tomoregulin as a growth factor signaling molecule (37, 38). These and our observations, therefore, suggest that C29 acts by inactivating tomoregulin-1. In view of 2 recent trials in prostate cancer targeting tomoregulin, it is of the utmost importance to dissect signaling pathways mediated by tomoregulin (39, 40).

In conclusion, our results, together with the recently published data, suggest that the novel type I transmembrane protein tomoregulin-1 may function as a growth factor (receptor), whereas inhibition of its function may lead to

apoptosis with Ser/Thr-dependent kinases in the downstream signaling pathway. Studies with RNAi will be performed to verify this point.

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