ELEVATED LEVEL OF gas3 GENE EXPRESSION IS CORRELATED WITH G0 GROWTH ARREST IN HUMAN FIBROBLASTS

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The gas3/PMP22 gene product is a dual function protein, involved in both peripheral nerve myelination and cell proliferation. gas 3/PMP22 is highly expressed in myelinating Schwann cells and is required for normal PNS development. In addition, a more general function for gas3 is suggested by its expression in non-neural tissues and upregulation by growth arrest in cultured rodent fibroblasts. In the present work, the expression of the gas3 gene has been studied in human fibroblasts. We have confirmed that gas3 mRNA is upregulated when cells are serum starved or grown to high cell density (G0 arrest). When quiescent cells were stimulated by serum or platelet-derived growth factor-BB (PDGF-BB), gas3 mRNA was down regulated. In contrast, we found that the expression of gas3 mRNA was neither upregulated in senescent cells nor in cells arrested in G1 using Lovastatin. Thus, high expression of gas3 is not related to growth inhibition in general, but more probably to the G0 growth arrest state. Furthermore, we found that in two malignant fibrous histiocytoma cell lines, gas3 expression was lower than in normal fibroblasts, suggesting an altered regulation of the gas3 gene in transformed cells.

INTRODUCTION

Normal cellular growth is regulated by positive as well as negative controls. Disruption of the balance between these systems can lead to unregulated growth and neoplastic transformation. The control systems comprise, inter alia, growth factors, growth factor receptors, regulatory molecules along the signal transduction pathways, and cell cycle regulators. For instance, mitogenic factors such as platelet-derived growth factor (PDGF) stimulate entry into S-phase. G1/S transition is made permissible by phosphorylation of pRb through the action of cyclin dependent kinases (cdks), which are negatively regulated by cdk inhibitors such as p16 and p21 (Granà et al., 1995).

Growth of normal anchorage-dependent cells ceases as the cultures reach saturation density (density-dependent inhibition of growth). This type of growth inhibition is, in several respects, similar to the growth arrest induced by depriving cells of growth factors; under both circumstances the cells enter an 'out of cycle' growth arrest state, referred to as G0 (Baserga, 1985). A nother important regulatory system becomes operative at high passage levels when the proliferative capacity gradually decreases. This phenomenon is known as cellular senescence or phase III (Hayflick and Moorhead, 1961).

A number of genes are specifically expressed in quiescent or senescent cells (Coppock et al., 1993; Gusticich and Schneider, 1993; Pignolo et al., 1993; Noda et al., 1994). Growth arrest-specific (gas) genes were identified in growth inhibited NIH3T3 cells (Schneider et al., 1988). In these cells, gas mRNAs appear after serum starvation or density-dependent inhibition of cellular growth and disappear as serum starved cells are stimulated to re-enter the cell cycle (Del Sal et al., 1992; Manfioletti et al., 1990).
The gas3 gene codes for a 22-kDa transmembrane glycoprotein and was also isolated by two independent laboratories as a gene whose expression is downregulated in rat Schwann cells during nerve regeneration (Spreyer et al., 1991; Welcher et al., 1991). The gas3 gene is highly expressed in differentiated Schwann cells and its protein product (also denoted PM P22 for peripheral myelin protein 22) is assembled into the peripheral myelin sheaths. Mutations involving the gas3 gene have been detected in hereditary demyelinating neuropathies in mice (Suter et al., 1992) and humans (Patel et al., 1992; Suter et al., 1994), i.e. diseases which are characterized by hypomyelination and continuous Schwann cell proliferation. These findings, together with the growth arrest-specific expression pattern in mouse fibroblasts have led to the suggestion that the gas3/PM P22 protein may have two biological functions, one related to myelin formation and another to cell growth (Lemke, 1993; Bosse et al., 1994). This idea is supported by the finding that gas3/PM P22 expression is regulated by two alternatively used promoters, located immediately upstream of two alternative 5' exons (exon 1A and 1B). Thus, two transcripts containing alternative 5' untranslated regions are differentially expressed in a tissue-specific manner, where one (containing exon 1A) dominates in Schwann cells and the other (containing exon 1B) in non-neuronal tissues and cultured fibroblasts (Suter et al., 1994).

With the aim of further characterizing the function of gas3 in human cells, we have studied its expression in human neonatal foreskin fibroblasts under different growth conditions. We have found that gas3 mRNA is highly expressed in human neonatal fibroblasts rendered quiescent by serum starvation or high cell density. When quiescent cells re-enter the cell cycle after addition of serum or PDGF, gas3 mRNA is downregulated. We also studied the expression of gas3 mRNA in cells growth inhibited by other mechanisms, i.e. in high passage (senescent) cells and cells arrested in G1 by treatment with Lovastatin. Our data suggest that the expression of the gas3 gene correlates specifically with the G0 growth arrest state in normal human fibroblasts. Since gas3 may have a function in maintaining a growth arrest state (Zoidl et al., 1995), we determined whether gas3 mRNA was expressed differently in cells with impaired growth regulation. Therefore, gas3 mRNA levels were investigated in cell lines derived from a malignant fibrous histiocytoma (fibrosarcoma). We found that these cell lines failed to upregulate gas3 mRNA at growth arrest.

**MATERIALS AND METHODS**

**Cell culture**

The human skin fibroblast strains GM 1518, AG 1523 (both of donor age 3 months) and GM 1680 (donor age 70 years) were purchased from the Human Mutant Cell Repository, Camden, NJ. The cells were grown in Eagle’s minimal essential medium, supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 100 units/ml and streptomycin 50 µg/ml). The cell lines U-2149 and U-2197 were established in our laboratory (Genberg et al., 1989), and are derived from two different recurrences of a malignant fibrous histiocytoma of a 72-year-old patient. Cells were seeded in 100-mm dishes for RNA preparations. To serum starve GM 1518, AG 1523 and GM 1680, the medium was changed to MCDB 104 medium containing antibiotics as above. For serum starvation of U-2149 and U-2197 cells Eagle’s MEM with antibiotics and 0.1% FCS was used. For constructions of growth curves, duplicate cell cultures were trypsinized at various time points after seeding, and counted in a Coulter counter. G1 phase synchronized cells were obtained by treating proliferating cells with Lovastatin (Merck, Sharp & Dohme Research Laboratories, PA) at 5 µM for 36 h, with one medium change after 18 h.

**RNA preparation and Northern blot analyses**

Total RNA was prepared by the LiCl/urea method (Auffray and Rougeon, 1980). Five to ten milligrams of RNA was denatured at 65°C in 50% formamide, 6.6% formaldehyde and 20 mM EDTA and separated on 0.8% agarose gels containing 2.2% formaldehyde. The RNA was then transferred to hybond C membranes (Amersham, U.K.) by capillary blotting. Filters were baked under vacuum for 2–4 h and prehybridized for 4 h in 42°C in 20% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt’s solution (0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovin serum albumin) and 250 mg/ml denatured salmon sperm DNA. gas3 (full length cDNA, Edomi et al., 1993) and GAPDH (Tso et al., 1985) cDNA fragments were labelled by the megaprime labelling kit from Amersham and hybridization took place at 42°C overnight in the above solution but with 50% formamide. Filters were washed at 65°C in 2 × SSC, 0.5% for 30–60 min and in 0.1 × SSC, 0.1% SDS for 0–20 min. The filters were then exposed to phosphoimaging plates (FUJI-type BAS-3) for 12–48 h and the images obtained were...
processed in the program Fujix BAS 2000 EWS Software version 2.1 1991. The signals obtained from gas3 hybridizations were scanned and normalized to the GAPDH hybridization signals. Figures show a representative of three or more experiments.

Autoradiographical analysis of \[^{3}H\] thymidine incorporation

Cells were incubated with 1 \(\mu\)Ci/ml of \[^{3}H\]thymidine (Amersham, specific activity 5 Ci/mmol) for 48 h after which the cells were fixed in methanol/acetic acid (3:1). The dishes were exposed to K odak NTB2 emulsion for 1 week at 4°C and developed. The cells were then stained with Giemsa and the percentage of labelled nuclei was determined. 400–500 cells per culture were analysed.

Cell cycle analysis

Cell cycle distribution was determined by propidium iodide staining of DNA. Briefly, cells were trypsinized, pelleted, redissolved in a solution containing 250 mM sucrose, 40 mM trisodium citrate and 5% dimethylsulfoxide (DMSO), and stored at -70°C. For FACS analysis, the samples were thawed, treated with trypsin and RNAse and finally stained with propidium iodide. The samples were subjected to analysis on FACSort (Becton Dickinson) flow cytometer. Calculations of cell cycle distribution were made by the MacCycle program.

RESULTS

High expression of gas3 in growth-arrested human fibroblasts

An upregulation of gas3 expression after growth arrest has been observed in the murine NIH 3T3 cell line (Schneider et al., 1988; Manfioletti et al., 1990). To investigate if the expression of gas3 is related to growth arrest in untransformed human fibroblasts, Northern blot analyses were performed. We studied gas3 mRNA levels in cells that were growth arrested in the G0 phase of the cell cycle by serum starvation of high cell density. AG1518 neonatal fibroblasts were seeded in medium containing 10% FCS, and after 2 days the cells were serum starved by changing to serum free MCDB medium. RNA was extracted before starvation (d0) and at 1, 2, 3 and 4 days after medium change. The gas3 mRNA level started to increase after 1 day of serum starvation (Fig. 1(a)). The increase in gas3 expression correlated to the decrease in number of proliferating cells as measured by \[^{3}H\]thymidine incorporation (Fig. 1(b)). To investigate if gas3 expression increased during density-dependent growth inhibition, we examined the mRNA levels in cells that were seeded very sparsely and then continuously grown in medium containing 10% FCS. Cells were harvested for RNA preparation at the indicated time points, and the cell numbers were determined. The gas3 mRNA level began to increase after 5 days as shown in Figure 1(c). The growth curve (Fig. 1(d)) indicates that the increase in gas3 expression correlated with the decline in proliferation.

Kinetics of gas3 downregulation

gas3 expression was analysed when serum starved cells were stimulated to enter the cell cycle. Subconfluent AG1518 cells were serum starved for 2 days, after which medium containing 20% FCS was added. At different time points, RNA was prepared. As shown by Fig. 2(a), gas3 mRNA expression was markedly downregulated after serum stimulation. We also investigated the levels of gas3 mRNA in sparse and density inhibited cells after serum starvation. The dense cultures were obtained by seeding the cells 2 weeks before the experiment. Sparse cultures were seeded 2–3 days before changing to serum free MCDB medium. After 2 days of serum starvation the cell cultures were treated with 10 ng/ml of PDGF-BB for different time periods. As presented in Fig. 2(b), the gas3 expression was reduced in both dense and sparse cultures after mitogen stimulation.

Together these experiments demonstrate that gas3 expression is rapidly downregulated after mitogen-stimulation of quiescent cells, reaching very low levels at 14 h, which is prior to initiation of S phase in AG1518 cells (C. Karlsson, unpublished).

Low gas3 expression in cells treated with Lovastatin

The previously described experiments revealed a correlation between gas3 mRNA expression and growth arrest. To further investigate if this correlation was seen in cells arrested by other mechanisms than serum starvation or density-dependent growth inhibition (assumed to induce arrest in G0), we used Lovastatin. Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.
Fig. 1. (a) Effect of serum starvation on gas3 mRNA levels. Sparse cell cultures of AG1518 were serum starved for different time periods after which RNA was prepared and analyzed by Northern blotting. (b) \(^{3}H\)Thymidine incorporation of cell cultures during serum starvation. Cells in the above experiment were given \(^{3}H\)Thymidine for 24 h before each harvest of RNA, and the dishes were subjected to autoradiography. The values shown are based on counting 500 cells/time point. (c) gas3 expression during density-dependent growth inhibition. AG1518 cells were seeded sparsely and grown in 10% FCS for several weeks with continuous medium change. RNA was extracted at the indicated days after seeding. (d) Growth curve. Each time cells were harvested for RNA preparations, cell numbers were determined by counting duplicate cultures.
This enzyme regulates the formation of mevalonate which is required for cell proliferation. Lovastatin has been shown to synchronize various cell types in the early G1 phase of the cells cycle (Keyomarsi et al., 1991). To obtain a G1 arrested cell population, Lovastatin was added to subconfluent cell cultures, growing in the presence of 10% FCS. Incubation of AG1518 cells with Lovastatin at a concentration of 5 μM for 36 h generally resulted in 85–95% of the cells to arrest with a G1 DNA content as determined by FACS analysis (data not shown). RNA was prepared from cycling cells, cells treated with Lovastatin, and cells serum starved for 2 days in MCDB medium. Northern blot analysis revealed that gas3 gene expression levels were considerably lower in Lovastatin arrested cells than in cells made quiescent by serum starvation (Fig. 3).

**Altered gas3 expression in senescent cells**

We also studied the mRNA levels in cell cultures which had ceased to divide due to high replicative age (senescent cells). GM1523 human neonatal fibroblasts of passage 35–40 were used. These cells did not reach confluency after 3 weeks in 10% FCS and were thus considered senescent. Cells of high and low passage were serum starved in MCDB medium for two days and then stimulated with Eagle's MEM containing 10% FCS. As shown in Fig. 4, gas3 is expressed at lower levels in serum starved senescent cells. After serum stimulation gas3 expression decreased to the levels in serum stimulated young cells.

**Reduced gas3 expression in two fibrosarcoma cell lines**

Considering the correlation between a G0 arrest state and gas3 expression we found it interesting to investigate whether abnormally growing cells express similar levels of gas3 as normal cells. For these experiments we used two fibroblast derived tumour cell lines (malignant fibrous histiocytomas;
fibrosarcomas). The U-2149 and U-2197 cell lines are derived from two different recurrences of the tumour from the same patient, U-2197 being more malignant. As an age-matched normal fibroblast control we used GM1680. Cells were serum starved for 2 days in Eagle’s MEM containing 0.1% FCS and then stimulated for 24 h with the same medium containing 10% FCS, followed by RNA extraction. The gas3 gene was less upregulated in both cell lines compared to the normal fibroblast controls during serum starved conditions (Fig. 5(a)). The percentages of cells incorporating [3H]thymidine under these conditions are shown in Fig. 5(b).

**DISCUSSION**

We have shown that the gas3 mRNA level increases dramatically when normal foreskin fibroblasts (AG1518) are rendered quiescent by serum starvation or by growth at high cell density. Addition of serum or PDGF-BB, a potent fibroblast mitogen, led to a marked decrease in gas3 mRNA expression. These results show that expression of gas3 is cell cycle dependent, and agree with previous studies in NIH 3T3 cells correlating high expression of gas3 with G0 arrest (Manfioletti et al., 1990; Schneider et al., 1992). Our finding that PDGF-BB cause a downregulation of gas3 mRNA levels in serum deprived cultures is in contrast to a report by Kletsas and coworkers, where no downregulation of the gas3 mRNA was seen after PDGF-stimulation in growth arrested human embryonic fibroblasts (Kletsas et al., 1995). These authors however observed a reduction in gas3 mRNA levels after TGF-β treatment which was not detectable in our cells system (data not shown). These differences could be due to the fact that different donor ages were used, i.e. of neonatal vs embryonic origin.

We found that gas3 mRNA expression was downregulated after mitogen stimulation of density inhibited cultures which are essentially refractory to mitogenic stimulation; only a small fraction of dense cells entered S phase under the conditions used in the experiments (data not shown). We have recently shown that early growth factor inducible
genes such as c-fos and c-myc are induced in density arrested human fibroblasts whereas the late genes cdc25A, E2F 1 and cyclin A are not (Afarkhite et al., 1998). Taken together these findings suggest that mitogenic stimulation of density inhibited cells at least partially releases them from the G0 block although the cells are prevented from further progression through G1.

Senescent cells do not enter S phase after growth factor stimulation. The growth inhibition is mediated by the inability of the cells to phosphorylate the R B protein, probably through increased levels of the cdk inhibitor p21 and p16 (Noda et al., 1994; Alcorta et al., 1996). Thus, in serum containing medium, the cells are blocked in late G1. Furthermore it has been suggested that senescent cells are unable to enter a true G0 arrest state even when forced to grow at high cell density (Pignolo et al., 1994). Our finding that serum starved senescent cells express lower levels of gas3 mRNA than young cells, agrees with the idea that senescent cells are arrested in a state that differs from the G0 arrest state of young cells.

The drug Lovastatin has been found to cause a reversible growth arrest in a number of cell types. The G1 phase specificity of this cell cycle arrest was demonstrated by a shorter lag period before the onset of DNA synthesis in Lovastatin treated cells compared to serum deprived cultures and by the presence of the proliferation associated marker, Ki 67 in Lovastatin treated cells (Keyomarski et al., 1991). In the present study, Lovastatin-treated human fibroblasts expressed levels of gas3 mRNA comparable to those in cycling cells. Thus, synchronization of cells in the G1 phase of the cell cycle does not lead to the increase in gas3 expression observed in serum starved, G0 arrested cells.

Taken together, the results from the experiments on senescent cell cultures as well as the Lovastatin treated cells, indicate that high expression of the gas3 gene is not related to growth arrest in general but rather is specific for a G0 growth arrest.

We have also found that gas3 mRNA levels were reduced in the two tumor cell lines compared to normal fibroblasts of the same donor age (Fig. 5 (a)). The inability of the fibrosarcoma cell lines to upregulate gas3 mRNA after serum starvation could partly be explained by the fact that some cells in the population did not arrest (18% for U-2197 and 14% for U-2149, see Fig. 5 (b)) under these conditions, or that the transformed cell lines are unable to enter a true G0 state. Nevertheless it is an interesting finding since gas3 have been proposed to have a growth retarding effect in other cell systems. In a study by Zoidl et al. (1995) both of the gas3 transcripts were found to be upregulated in quiescent rat Schwann cells. Furthermore, overexpression of gas3/PM P22 delayed the entry into S phase after serum stimulation. Expression of an antisense gas3/PM P22 construct increased the proportion of cells in the G2 and S phases compared to control cells after stimulation, which suggests that gas3 may have a functional role in maintaining the quiescent state. Whether the reduced levels of gas3 mRNA are conferring a growth advantage on the fibrosarcoma cells remains to be elucidated. There is, however, a previous study describing a markedly decreased expression of gas3 in urethan-induced mouse lung tumours which could imply a role for gas3 in transformation and tumorigenesis (Re et al., 1992).

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REFERENCES


