Suppression of metastasis-associated S100A4 gene expression by gamma-interferon in human colon adenocarcinoma cells

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Summary S100A4 belongs to the S100 subfamily of calcium-binding proteins and has been suggested to be directly involved in invasion and metastasis of rodent and human tumour cells. The present study demonstrates that interferon gamma (IFN-γ), but not IFN-α and IFN-β, down-regulates the S100A4 mRNA level in colon adenocarcinoma WiDr cells in time- and dose-dependent manners. The effect was not associated with any cytotoxicity and was specific for the S100A4 mRNA, since the levels of the S100A6 and GAPDH mRNAs were not significantly affected by the treatment. IFN-γ also strongly suppressed the S100A4 mRNA expression in HT-29 cells, but weakly in Colo201 cells. Flow cytometric analysis revealed that the level of the IFN-γ receptor expression in Colo201 cells was lower than that in WiDr and HT-29 cells, suggesting that the suppression of the S100A4 expression by IFN-γ depends on the amount of cell surface IFN-γ receptor protein. IFN-γ had no effect on the transcription rate of the S100A4 gene but reduced the stability of the S100A4 mRNA. WiDr cells treated with IFN-γ showed reduced motile ability, further supporting the assumption that the S100A4 gene product is involved in controlling cell motility.

Keywords: IFN-γ; S100A4; IFN-γ-receptor; motility; colon carcinoma

S100A4, also known as CAPL, pEL98, mts1, p9Ka, 18A2, 42A and FSP1, is a member of the S100 subfamily of calcium-binding proteins (Hilt and Kligman, 1991; Celio et al, 1996; Schüler and Heinzmann, 1996). Its expression has been shown to be associated with cell immortalization (Goto et al, 1988), cell growth (Jackson-Grusby et al, 1987), differentiation of myoepithelial cells (Barracough et al, 1987) and promyelocytic leukaemia cells (Takenaga et al, 1994b), fibrogenesis (Strutz et al, 1995) and tumour progression (Grigorian et al, 1996). In addition, several lines of evidence indicate that S100A4 is directly involved in tumour cell invasion and metastasis. Ebralitze et al reported that the expression of S100A4 was elevated in metastatic tumour cells (Ebralidze et al, 1989). We and others have shown that the increased expression of S100A4 in tumour cells by introduction of the S100A4 gene resulted in an enhancement of cell motility and invasive and metastatic potential of tumour cells (Davies et al, 1993; Parker et al, 1994; Takenaga et al, 1994a). On the other hand, reduction of the expression of S100A4 by means of antisense S100A4 RNA or anti-S100A4 ribozyme in high-metastatic tumour cells resulted in the suppression of their invasive and metastatic capabilities (Grigorian et al, 1993; MeLandrino et al, 1996; Takenaga et al, 1997a). Although the precise functions of the S100A4 protein are not fully understood, recent studies implicate that the protein interacts with cytoskeletal proteins such as non-muscle tropomyosins (Takenaga et al, 1994c, 1994d) and myosin (Krajcevskia et al, 1994; Ford and Zain, 1995) in a Ca²⁺-dependent manner and thereby modulates cell motility (Takenaga et al, 1994a, 1994d; Ford and Zain, 1995). Importantly, several studies have shown that S100A4 may also play a role in invasion and metastasis of human tumours including breast (Pedrocchi et al, 1994; Ilg et al, 1996) and colorectal carcinomas (Ilg et al, 1996; Takenaga et al, 1997b).

Metastasis comprises a complex series of interactions between tumour cells and their environment (Radinsky, 1995). At each step, tumour cells are exposed to a variety of local factors such as hormones, growth factors and cytokines. These factors undoubtedly modulate the metastasis-associated properties of tumour cells such as growth potential, secretion of type IV collagenolytic activity, cell migration, cell adhesion and natural killer (NK) cell sensitivity. Since it is largely unknown whether the S100A4 expression in tumour cells is also influenced by such factors, the effects of various growth factors and cytokines on the expression of S100A4 in human colon adenocarcinoma cells were examined. In this study, the fact that interferon gamma (IFN-γ) efficiently suppresses the S100A4 expression and reduces the motile ability in the cells is demonstrated.

MATERIALS AND METHODS

Reagents

Recombinant human IFN-α (specific activity; 3 × 10⁶ IU mg⁻¹) and IFN-γ (specific activity; 3 × 10⁷ IU mg⁻¹) were purchased from Pepro Tech EC Ltd (London, UK). Human IFN-β (specific activity; 1 × 10⁶ IU mg⁻¹) and mouse monoclonal anti-human IFN-γ receptor (clone GIR-208) were obtained from Sigma Chemicals Co. (St Louis, MO, USA). Recombinant human tumour necrosis factor-α (TNF-α) was purchased from Gibco BRL (Grand Island, NY, USA).
Cell culture

Human colon adenocarcinoma cell lines, WiDr, Colo201 and HT-29, were obtained from the American Type Culture Collection (Rockville, MD, USA). They were cultured in RPMI-1640 medium supplemented with 10% heat (56°C, 30 min)-inactivated fetal bovine serum, 100 ng ml⁻¹ streptomycin and 100 units per ml penicillin.

Western blot analysis

Cells were lysed in extraction buffer (150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EGTA and 1 mM phenylmethylsulphonyl fluoride) (Takenaga et al, 1994). Cell lysates were centrifuged at 10 000 g for 10 min, and the supernatant was used for immunoblot analysis. Immunoblot analysis was carried out as described previously (Takenaga et al, 1994) using anti-S100A4 polyclonal antibody and an enhanced chemiluminescence Western blotting detection kit (Amersham).

Northern blot analysis

Twenty micrograms of RNA which was extracted with guanidinium thiocyanate were electrophoresed on 1% agarose gel containing formaldehyde and transferred to nylon filters (Sambrook et al, 1989). Blots were hybridized with a ³²P-labelled human S100A4, S100A6 (a gift from Dr DT Denhardt) or glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) cDNA which was prepared by the random primer method (Sambrook et al, 1989). Filters were finally washed at 50°C in 0.2 x saline–sodium citrate (SSC) containing 0.1% sodium dodecyl sulphate (SDS).

Evaluation of S100A4 mRNA stability

Untreated cells and cells treated with 300 IU ml⁻¹ IFN-γ for 10 h were incubated with 5 µg ml⁻¹ actinomycin D for up to 12 h to block further transcription. Total RNA was extracted and subjected to Northern blot analysis. The blots were hybridized with either an S100A4 cDNA probe or a human GAPDH cDNA probe. After quantifying the intensities of bands by scanning the autoradiograms, the levels of S100A4 mRNA were normalized to those of GAPDH mRNA.

RESULTS

Effect of IFN-γ on the S100A4 mRNA expression in human colon adenocarcinoma WiDr cells

The effects of IFN-γ and TNF-α on the expression of S100A4 mRNA were examined in WiDr cells, which are reported to express S100A4 (Takenaga et al, 1997b). Treatment of the cells with 300 IU ml⁻¹ IFN-γ for 48 h resulted in a striking suppression of the S100A4 expression (Figure 1A). However, the expressions of S100A6 and GAPDH mRNAs were not significantly altered by the IFN-γ treatment (Figure 1A). IFN-α (300 IU ml⁻¹), IFN-β (300 IU ml⁻¹) and TNF-α (10 ng ml⁻¹) showed no effect on the S100A4 expression (Figure 1A,B). These results indicate that the suppression of S100A4 is specific for IFN-γ. The inhibition of the S100A4 expression by IFN-γ was clearly detectable as early as 10 h after the treatment (Figure 2A) and at the concentrations above 200 IU ml⁻¹ (Figure 2B). Western blot analysis also revealed a reduction in the S100A4 protein in IFN-γ-treated WiDr cells (Figure 1C). The effects of interleukin 1 (IL-1), IL-6 and various growth factors such as fibroblast growth factor, epidermal growth factor, hepatocyte growth factor and insulin growth factors on the S100A4 expression were also investigated. The results showed that they neither stimulate nor inhibit the S100A4 expression (data not shown).

Effect of IFN-γ on S100A4 expression and cell surface IFN-γ-R expression in colon adenocarcinoma cells

To examine whether IFN-γ suppresses the expression of S100A4 in other colon adenocarcinoma cells, Colo201 and HT-29 cells, both of which express S100A4 (Takenaga et al, 1997b), were incubated with 300 IU ml⁻¹ IFN-γ for 48 h. The results in Figure 3 show that IFN-γ significantly suppressed the S100A4 expression in HT-29 cells as observed in WiDr cells, but only weakly in Colo201 cells. The reason might be due to the lower expression of cell surface IFN-γ-R in Colo201 cells than in WiDr and HT-29 cells. To address this question, the cells were incubated with an anti-IFN-γ-R monoclonal antibody followed by incubation with a dilution of mouse anti-human IFN-γ-R monoclonal antibody, washed, and then incubated for 30 min with a 1:200 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody in PBS/BSA. After washing, cells were fixed with 3% paraformaldehyde and then analysed by fluorescence-activated cell sorting (FACSCAN, Beckton Dickinson, Mountain View, CA, USA).
fluorescein-conjugated secondary antibody and analysed by fluorescence-activated cell sorting. The results showed that the expression of cell surface IFN-γ-R was indeed lower in Colo201 cells than in WiDr and HT-29 cells (Figure 4).

Effects of IFN-γ on S100A4 gene transcription and S100A4 mRNA stability in WiDr cells

The molecular mechanism by which IFN-γ down-regulates the steady-state level of S100A4 mRNA was explored by examining the effect of IFN-γ on the S100A4 gene transcription. In vitro transcription analyses showed that the transcriptional rate of S100A4 mRNA as well as that of GAPDH mRNA in IFN-γ-treated cells was indistinguishable from that in untreated cells (Figure 5A). The decay rate of S100A4 mRNA was next compared between WiDr cells and the cells treated with IFN-γ. As shown in Figure 5B, the level of S100A4 mRNA in control cells did not change during a 12-h incubation period with actinomycin D, indicating that the S100A4 mRNA is highly stable as reported previously (Grigorian et al, 1994). On the other hand, the S100A4 mRNA in IFN-γ-treated cells was decayed more rapidly than that in untreated cells. These results suggest that the down-regulation of the steady-state level of the S100A4 mRNA in WiDr cells treated with IFN-γ was partly due to the reduced S100A4 mRNA stability.

Effect of IFN-γ on cell motility of WiDr cells

WiDr cells were pretreated with 300 IU ml⁻¹ IFN-γ for 24 h and then subjected to the motility assay in the presence of IFN-γ for another 24 h. As shown in Figure 6, the motile ability of WiDr cells treated with IFN-γ was significantly lower than that of untreated cells.

DISCUSSION

The present study has shown that IFN-γ, but not IFN-α and IFN-β, suppressed the expression of S100A4 gene in colon adenocarcinoma cells. Several lines of evidence support that the suppression is not due to its cytotoxicity. First, the expressions of S100A6 and
GAPDH mRNAs were not significantly suppressed by IFN-γ. Second, TNF-α (10 ng ml⁻¹), which inhibited the cell growth to a similar extent as IFN-γ (300 units ml⁻¹) (approximately 20%) did not suppress the S100A4 mRNA expression. Third, the suppression of the S100A4 mRNA expression was observed as early as 10h after the IFN-γ treatment, a time when the cells were fully viable.

IFN-γ has been shown to modulate expressions of a variety of genes (Sen and Lengyel, 1992). The mechanisms for the down-regulation of genes caused by IFN-γ are less understood than those for the up-regulation of genes in which the sequences in the 5'-flanking regulatory region, as well as transcription factors, are implicated (Sen et al, 1992). The expression of type II collagen gene has been shown to be down-regulated by IFN-γ, primarily at the transcriptional level (Goldring et al, 1994). On the other hand, genes coding for c-fos (Radzich and Varesio, 1991), c-myc (Harel-Bellan et al, 1988), transferrin receptor (Bourgeade et al, 1992) and cystic fibrosis transmembrane conductance regulator (Besançon et al, 1994) have been post-transcriptionally modulated by IFN-γ. To examine the mechanisms by which IFN-γ suppresses the S100A4 gene expression, the transcriptional rate of the S100A4 gene and the mRNA stability were examined. The results showed that IFN-γ did not affect its transcription rate but reduced the mRNA stability, indicating that the expression of the S100A4 gene expression in human colon adenocarcinoma cells is down-regulated by IFN-γ at the post-transcriptional level. The mechanism by which IFN-γ reduces the S100A4 mRNA stability is an interesting issue to be solved. Since it has recently been demonstrated that the methylation of S100A4 gene is crucial for the gene expression in human colon adenocarcinoma cell lines (Nakamura and Takenaga, 1998), the DNA methylation status of the S100A4 gene before and after the treatment of WiDr cells with IFN-γ was also examined. However, there was no detectable difference in the degree of DNA methylation between untreated and IFN-γ-treated WiDr cells (data not shown).

Transfection of v-src-transformed 3Y1 cells and mouse mammary adenocarcinoma CSMLO cells with S100A4 gene resulted in an increased cell motility (Takenaga et al, 1994a; Ford et al, 1995). Conversely, the expression of antisense S100A4 mRNA in S100A4-expressing Lewis lung carcinoma cells lowered cell motility (Takenaga et al, 1997a). These results collectively support the assumption that S100A4 participates in regulating cell motility. The present study suggested that IFN-γ-treated WiDr cells became less motile than untreated cells through the suppression of the S100A4 expression. However, the involvement of other factors in the inhibition of cell motility of WiDr cells by IFN-γ cannot be neglected, because IFN-γ possesses a variety of biological effects.

Colo201 cells that express a lower level of IFN-γ-R than WiDr and HT-29 cells were more refractory to IFN-γ in terms of the inhibition of the S100A4 mRNA expression. This result suggests that the inhibition of the S100A4 mRNA expression by IFN-γ is mediated by cell surface IFN-γ-R. If this is the case, an attempt to
increase the cell surface IFN-γ-R in Colo201 cells may influence the sensitivity of the cells to IFN-γ. Since it has been reported that IL-1 and TNF-α are able to increase the cell surface IFN-γ-R expression in colon carcinoma cells (Raitano and Korc, 1993), the effects of IL-1 (1000 units per ml) and TNF-α (20 ng ml⁻¹) on the cell surface IFN-γ-R expression in Colo201 cells were preliminarily examined. It was found that IL-1 caused an approximately twofold increase in the expression of IFN-γ-R (data not shown). Colo201 cells were then simultaneously treated with IFN-γ and IL-1, and the S100A4 mRNA expression was measured. The results showed that IL-1 augmented the suppressive effect of IFN-γ on S100A4 expression (data not shown). Therefore, the combination treatment of colon adenocarcinoma cells with IFN-γ and IL-1 would be more effective in reducing the S100A4 mRNA expression and thereby in suppressing invasive and metastatic potential of colon adenocarcinoma cells.

Previous reports have shown that IFN-γ shows either a suppressing or an enhancing effect on metastasis of tumour cells. IFN-γ inhibits metastasis of mouse ovarian and lung tumour cells (Ossina et al, 1997), renal adenocarcinoma cells (Yanagihara et al, 1994) and human non-small-cell lung cancer cells (Masumori et al, 1995). By contrast, it enhances experimental metastatic potential of colon 26 adenocarcinoma cells (An et al, 1996) and B16 melanoma cells (Ramani and Balkwill, 1987). These results may be due to the time of treatment of the cells with IFN-γ, as suggested by some investigators (McMillan et al, 1987), or the properties of each cell line. Unfortunately, it is largely unknown whether IFN-γ influences invasiveness and metastasis of human colon adenocarcinoma cells. Therefore, the effects of IFN-γ on these phenotypes should be further clarified.

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