Increased Expression of Insulin-like Growth Factor I Receptor in Malignant Cells Expressing Aberrant p53: Functional Impact

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ABSTRACT

We investigated the functional impact of p53 on insulin-like growth factor I receptor (IGF-IR) expression in malignant cells. Using the BL-41tsp53-2 cell line, a transfectant carrying temperature-sensitive (ts) p53 and endogenous mutant p53 (codon 248), we demonstrated a drastic down-regulation of plasma membrane-bound IGF-IRs on induction of wild-type p53. However, a similar response was obtained by treatment of BL-41tsp53-2 cells expressing mutant ts p53 with a p53 antisense oligonucleotide. Thus, even if the negative effect of wild-type p53 predominates under a competitive condition, these data indicate that mutant p53 may be important for up-regulation of IGF-IR. To further elucidate this issue, three melanoma cell lines (BE, SK-MEL-5, and SK-MEL-28) that overexpressed p53 were investigated. The BE cell line has a “hot spot” mutation (codon 248) and expresses only codon 248-mutant p53. SK-MEL-28 has a point mutation at codon 145. SK-MEL-5 cells did not exhibit any p53 mutations, but the absence of p21Waf1 expression suggested functionally aberrant p53. Our data suggest that interaction with Mdm-2 may underlie p53 inactivation in these cells. Using p53 antisense oligonucleotides, we demonstrated a substantial down-regulation of cell surface expression of IGF-IR proteins in all melanoma cell lines after 24 h. This was paralleled by decreased tyrosine phosphorylation of IGF-IR and growth arrest and, subsequently, massive cell death was observed (this was also seen in BL-41tsp53-2 cells with mutant conformation of ts p53). Taken together, our results suggest that up-regulation of IGF-IR as a result of expression of aberrant p53 may be important for the growth and survival of malignant cells.

INTRODUCTION

Alterations of the p53 suppressor oncogene (TP-53) have been widely reported in tumor cell lines and malignant tumors in vivo (1, 2). TP-53 is localized on the short arm of chromosome 17 (17p13) and contains 393 codons and a domain with transcription-activating properties at the NH2-terminal. Under certain conditions (such as UV irradiation or exposure to chemical carcinogens) leading to DNA damage, wt p53 suppresses cell proliferation and prevents transformation (1, 2). This suppressor function is caused, at least in part, by decreased tyrosine phosphorylation of IGF-IR and growth arrest, and, functionally aberrant p53. Our data suggest that interaction with Mdm-2 may underlie p53 inactivation in these cells. Using p53 antisense oligonucleotides, we demonstrated a substantial down-regulation of cell surface expression of IGF-IR proteins in all melanoma cell lines after 24 h. This was paralleled by decreased tyrosine phosphorylation of IGF-IR and growth arrest and, subsequently, massive cell death was observed (this was also seen in BL-41tsp53-2 cells with mutant conformation of ts p53). Taken together, our results suggest that up-regulation of IGF-IR as a result of expression of aberrant p53 may be important for the growth and survival of malignant cells.

MATERIALS AND METHODS

Materials. A mouse monoclonal antibody against human IGF-IR (a1R-3), p21Waf1, and Bcl-2 were purchased from Oncogene Science. A polyclonal IGF-IR antibody (N-20), mouse monoclonal antibodies against human p53 (DO1), a mouse monoclonal antibody against Mdm-2 (including the p53-Mdm-2 complex), a monoclonal antibody against phosphotyrosine (PY99), and an antibody against actin (H-196) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A pan-CD44 (IM-7) monoclonal antibody was from the American Type Culture Collection (Manassas, VA). The proteasome inhibitor lactacystin was from Calbiochem (Darmstadt, Germany). Unless otherwise stated, all other reagents were from Sigma (St. Louis, MO).

Cell Lines. The human melanoma cell lines SK-MEL-5 and SK-MEL-28, ES cell line RD-ES, and the human p53-negative cell lines Saos-2 and HL-60 were obtained from American Type Culture Collection. BL41-tsp53-2 is an EBV-negative Burkitt lymphoma cell line carrying mutant p53 (codon 248) transfected with ts p53 mutant (p53Val135) with mutant conformation at 37°C and wt conformation at 32°C (13–15). The HDFs (GM08333) were obtained from the Coriell Institute of Medical Research. BE cells, which were established from a lymph node metastasis specimen from a patient with advanced malignant melanoma (16), were kindly provided by Prof. Rolf Kiessling (Karolinska Hospital, Stockholm, Sweden). SK-MEL-5, SK-MEL-28, BE and GM08333 cells were cultured in MEM supplemented with 10% FCS, HL-60 cells were cultured in Iscove’s modified Eagle’s medium supplemented with 15% FCS, Saos-2 cells were cultured in McCoy’s 5a medium supplemented with 20% FCS, RD-ES cells were cultured in RPMI 1640 supplemented with 10% FCS, and BL-41 cells were cultured in RPMI 1640 with 10% FCS.

Immunoprecipitation. The isolated cells were lysed as described elsewhere (17). Protein G Plus-A/G-agarose (0.15 µl) and 1 µg of antibody were added to 1 ml of protein material. After a 24-h incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2500 rpm for 15 min. The supernatant was discarded, and the pellet was washed. The material was then dissolved in sample buffer for SDS-PAGE.

SDS-PAGE and Western Blotting. Protein samples were dissolved in a sample buffer containing 0.0625 M Tris–HCl (pH 6.8), 20% glycerol, 2% SDS, 5278
bromphenol blue, and DTT. Samples corresponding to 50–100 μg of cell protein were analyzed by SDS-PAGE with a 7.5% or 10% separation gel essentially according to the protocol of Laemmli (18). Molecular weight markers (Bio-Rad) were run simultaneously. After SDS-PAGE, the proteins were transferred overnight to nitrocellulose membranes (Hybond; Amersham) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS (pH 7.5). Incubation with the appropriate primary antibody was performed for 1 h at room temperature. This was followed by washes with PBS and incubation with a biotinylated secondary antibody (Amersham) for 1 h. After incubation with streptavidin-labeled horseradish peroxidase, detection was made (Hyperfilm-ECL; Amersham). The films were exposed to Fluor-S (Bio-Rad).

Quantification of IGF-IR and p53 mRNA Levels. Total RNA was isolated from the cells using RNAzol according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Total RNA (500 ng) was reverse transcribed into cDNA. Five μl of the cDNA reaction were used to amplify IGF-IR (forward primer, 5’-GCCGAGGGGGAGCAGGG-3’, position 1028; reverse primer, 5’-GGTACCCTCGAGGCTTTAGA-3’, position 1559), β-actin (forward primer, 5’-CAGCGAGTACTTGCGCTCAGGAGG-3’; reverse primer, 5’-CACCGGAGTACTTGCGCTCAGGAGG-3’), and p53 (forward primer, 5’-CCGGTGAAAGTAGAT TATGTA-3’, position 799; reverse primer, 5’-CAAGGGCTTAC TGGGGCATGCTGGAGG-3’, position 1299). Competitive PCR for quantification of IGF-IR and p53 transcripts was performed as described elsewhere (19).

Antisense Experiments. AS-ODNs (5’-CCCTGCTCCCCCTGCTCC-3’) and S-ODNs (5’-GGAGCAGGGGGGAGCAGGGG-3’) to p53 were purchased from Pharmacia Biotech. AS-ODN is complementary to position 1071–1090 of exon 10 of the p53 mRNA (20, 21). Lipofectin (Life Technologies, Inc.) was used to deliver AS-ODNs to cultured cells. Because AS-ODN induces RNS cleavage and further degradation of target mRNA, we tested the specificity of the p53 AS-ODN using semiquantitative RT-PCR. RNA was isolated from SK-MEL-5 and SK-MEL-28 cells that had been treated with p53AS-ODN for 24 h. As shown in Fig. 1, the p53 transcript was dramatically decreased after treatment with AS-ODN. In contrast, no decrease was observed in the Lipofectin control or after treatment with S-ODN plus S-ODN.

p53 Mutation Analysis. DNA was isolated by standard methods. Exons 2–10 of human p53 were amplified from cellular DNA using a multiplex/nested PCR protocol (22). PCR products were sequenced directly by cycle sequencing with dye-labeled terminators (BigDye Terminators; Perkin-Elmer, Applied Biosystems, Foster City, CA). The sequences obtained were identified and aligned together with the wt sequence (obtained from BLASTN, National Center for Biotechnology Information) using the DNA analyzer program sequencer (Gene Codes).

Isolation of Plasma Membranes. Preparation of plasma membranes was performed essentially as described elsewhere (23). In brief, cells were harvested and homogenized. After a 10-min centrifugation at 600 × g (4°C), the pellet (containing unbroken cells, nuclei, and cytoskeleton) was discarded. The supernatant was then centrifuged at 17,300 × g for 30 min. The resulting pellet contains plasma membranes (23).

Assay of Cell Growth and Survival. For assay of DNA synthesis, cells cultured in 35-mm dishes were labeled with [3H]thymidine (1 μCi/ml, 5 Ci/mmol) during the last 4 h of the experiments. The acid-precipitable material was then taken for scintillation counting as described elsewhere (17). Proliferation of the cell lines, with the exception of HDFs and BL-41ts53-2, was measured by determining the number of cells attached to the plastic surface of duplicate 35-mm dishes. This was performed by microscopic counting of cells in several ink-marked areas on the dish bottom. By repeating the countings after specified time intervals, changes in the number of attached cells could be followed (24). In HDFs, cell growth was assayed by determining changes in cellular protein content, and in BL-41ts53-2 cells, cell growth was assayed by counting cells in a Burker chamber.

RESULTS

Effect of wt p53 on Expression of IGF-IR. We first investigated the effect of induction of wt p53 on IGF-IR expression at the cell surface using BL-41ts53-2 cells. These cells express endogenous mutant p53 (codon 248) and ts p53 with mutant conformation at 37°C (13). At 32°C, expression of ts p53 with a wt conformation is induced (13). Using an antibody directed to the M1, 130,000 kDa subunit of IGF-IR, we investigated the expression of IGF-IR at the cell surface by Western blotting, as described elsewhere (17). A strong IGF-IR signal was seen in cells cultured at 37°C but was deleted at 32°C (Fig. 2A). As a control, we investigated IGF-IR expression in parental BL-41 cells. As shown in the right panel of Fig. 2A, there were no differences in the IGF-IR signals between the two culture conditions in the parental cell line. Thus, introduction of wt p53 in cells normally expressing mutant p53 leads to
with HDFs and BL-41tsp53-2 cells (32°C), was investigated. As expected, the BL-41tsp53-2 cells expressed p21Waf1 on induction of wt p53 (Fig. 3C). In contrast, there was no detectable p21Waf1 in HDFs and SK-MEL-5. Immunofluorescence analysis demonstrated similar results (data not shown). The reason why HDFs did not express p21Waf1 is most likely due to the low p53 expression in these cells under normal growth conditions (compare with Fig. 3A). The absence of p21Waf1 expression in SK-MEL-5 suggests that the suppressor function of p53 is lost in these cells. A possible mechanism involved in inactivation of wt p53 could be association with Mdm-2.

In Fig. 3D, the expression of Mdm-2 in SK-MEL-5 is compared with Mdm-2 expression in ES cells, the p53-negative osteosarcoma cell line Saos-2, and HDFs. Whereas Mdm-2 expression was not detectable in HDFs and ES cells and was weak in Saos-2 cells, this protein was strongly expressed in SK-MEL-5 cells. We also investigated whether p53 was complex-bound to Mdm-2 in SK-MEL-5. This was assayed by immunoprecipitation using an antibody to Mdm-2, followed by Western blot analysis of p53. As shown in Fig. 3E, there is a strong p53 signal in SK-MEL-5 cells. These data suggest that inactivation of p53 in SK-MEL-5 can be due to association with Mdm-2.

Relationship between Aberrant p53 and IGF-IR Expression. To investigate whether the expression of aberrant p53 in BE and SK-MEL-5 cells (due to two different mechanisms) has any influence on the cell surface expression of IGF-IR, p53 AS-ODN was delivered to the cells.

As a control experiment, we first analyzed the effect of AS-ODN on IGF-IR expression of the p53-negative cell line HL-60. We confirm that AS-ODN had no effect on IGF-IR in these cells (Fig. 4, top panel). In the middle panels of Fig. 4, we show that a 24-h treatment with AS-ODN down-regulated both p53 and IGF-IR in BE and SK-MEL-5 cells. This effect was prevented on coincubation with p53 S-ODN. Lipofectin per se did not affect p53 or IGF-IR expression. In the bottom panels of Fig. 4, we show that the expression of three other proteins (Bcl-2, CD44, and β-actin) functioning as loading controls was not altered after treatment with AS-ODN. Our results suggest that inhibition of the expression of aberrant p53 leads to down-regulation of IGF-IR. AS-ODN consistently decreased the level of IGF-IR tran-
scripts (Fig. 5), suggesting that p53 may regulate IGF-IR at a transcriptional level. However, this decrease was limited to 50–60%. This could be explained simply by insufficient accuracy of the semi-quantitative RT-PCR method used. Alternatively, the observed 50–60% decrease in transcripts may be sufficient to block the expression of the IGF-IR protein. Another explanation is that the block of p53 may also inhibit IGF-IR expression at posttranscriptional levels.

The data presented in Fig. 4 strongly suggest that inhibition of p53 expression drastically reduces the number of IGF-IRs in BE and SK-MEL-5 cells. A Scatchard plot analysis would be helpful to prove this, but binding analysis could be disturbed by p53-mediated changes of expression of IGF-binding proteins. p53 has in fact been demonstrated to regulate IGF-binding protein 3 (26). Instead, we decided to investigate the effect of p53 inhibition on IGF-IR activity by measuring the level of tyrosine phosphorylation of the β-subunit. This experiment was carried out on SK-MEL-5 and SK-MEL-28 cells. SK-MEL-28 has a point mutation in codon 145 of p53 (27). This cell line also overexpressed p53 and responded by down-regulation of IGF-IR to p53 AS-ODN (data not shown). As shown in Fig. 6, antisense treatment almost deleted IGF-I-mediated tyrosine phosphorylation in both cell lines. This suggests that p53-mediated changes of IGF-IR expression have functional impact.

Effect of Proteasome Inhibitors on Expression of IGF-IR. It has been shown that Mdm-2-targeted degradation of wt p53 is blocked by proteasome inhibitors (28, 29). As shown in the bottom panel of Fig. 7, the proteasome inhibitor lactacystin, which inhibits all three types of proteasomes (30), moderately increased the expression of wt p53 in SK-MEL-5 (by almost 40% as assayed by densitometry) but did not increase the expression of mutant p53 in SK-MEL-28. In SK-MEL-5, this was correlated with a drastic decrease in tyrosine phosphorylation of IGF-IR (Fig. 7, top panel). In contrast, the level of phosphorylated IGF-IR in SK-Mel-28 was not significantly changed.

Effect of p53 AS-ODNs on Cell Growth and Survival. To evaluate the effect of p53 inhibition on cell proliferation, we treated several cell lines with p53 AS-ODNs. As shown, p53 AS-ODNs did not inhibit the growth of normal human fibroblasts (Fig. 8A). In contrast, the growth of BE, SK-MEL-5, and BL-41tsp53-2 [37°C (Fig. 8, B–D)] treated with AS-ODN for 24–72 h was blocked. Nevertheless, after 48 h, a massive cell loss was seen. Closer microscopic analysis revealed that the detached cells had undergone cell death (data not shown). The decrease in the effect of AS-ODN on BL-41tsp53-2 after a 72-h treatment is probably due to degradation of AS-ODN. The effect on DNA synthesis, as assayed by incorporation of [3H]thymidine, was also investigated. A 24-h exposure to p53 AS-ODN drastically decreased the rate of DNA synthesis in BE, SK-MEL-5, and SK-MEL-28 cells (Fig. 8, E, F, and H) but not in p53-negative HL-60 cells (Fig. 8G).

Finally, we compared the effects of p53 AS-ODN with those of other inhibitors of IGF-IR, i.e., dI3-R (which inhibits the binding of IGF-I to IGF-IR; Ref. 31) and lovastatin, on the growth of BE and SK-MEL-5 cells. It was demonstrated previously that lovastatin blocks cell surface expression of IGF-IR and that this mechanism strongly contributes to lovastatin-induced growth arrest in melanoma cells (17, 24, 32). As demonstrated in Fig. 9, all three agents caused inhibition of DNA synthesis, growth arrest, and cell death.

DISCUSSION

The promoter region of the IGF-IR gene lacks TATA and CAAT elements, and, similar to other growth-related promoters, it has a high CG content (33). In addition, the promoter region of the IGF-IR gene contains multiple potential binding sites for several oncoproteins and tumor suppressors (33). Recently, evidence was provided that p53 interacts with the IGF-IR promoter (12).

In our present study, we investigated the functional impact of p53 on IGF-IR and IGF-IR-related events (cell growth and survival) in human malignant cells. To evaluate the effect on IGF-IR, we measured the amounts of IGF-IR at the cell surface in three different experimental systems. These involved: (a) a cell line with mutant p53 transfected with ts p53; (b) two malignant melanoma cell lines overexpressing p53 with point mutations, one of which only expressed mutant p53; and (c) a malignant melanoma cell line expressing functionally aberrant wt p53. We could demonstrate that induction of functionally normal wt p53 resulted in down-regulation of IGF-IR at the cell surface. This is in line with the observation of Werner et al. (12) showing that wt p53 represses the transcription of the IGF-IR gene. Their study was performed on p53-negative cells transfected with wt or mutant p53 cDNA, and the transcriptional effect was assayed using a construct carrying a luciferase reporter gene driven by the IGF-IR promoter (12).
To elucidate whether expression of functionally aberrant p53 could influence the expression of IGF-IR, the three malignant melanoma cell lines and BL-41 tsp53-2 cells, all of which overexpressed IGF-IR at the cell surface, were investigated. In SK-MEL-5, which did not exhibit any point mutations, Mdm-2 was overexpressed and coimmunoprecipitated with p53. This indicates that p53 is inactivated due to complex binding to Mdm-2. Anti-sense oligonucleotides against p53 resulted in a drastic downregulation of IGF-IR expression at the cell surface of all four cell lines. This was accompanied by reduced tyrosine phosphorylation of IGF-IR, a drastic decrease in DNA and cell replication, and, ultimately, by massive cell death. Similar responses were achieved by blocking the IGF-I pathway through other means. Our results suggest that IGF-IR expression can be a gain of function mechanism of aberrant p53 in malignant cells. This mechanism might help to protect malignant cells from apoptosis. A study by Prisco et al. (34) showing that 32D murine hemopoietic cells with constitutively expressed IGF-IR were resistant to the apoptotic effect of wt p53 after interleukin-3 withdrawal supports this notion.

It has been shown that mutant p53, in contrast to wt p53, stimulates the transcriptional activity of the IGF-IR gene (12). However, according to our present study, p53 does not need to be mutated to increase IGF-IR expression. It seems that expression of functionally aberrant p53, i.e., p53 lacking its suppressor function (through point mutations or interaction with Mdm-2) may be of importance. This notion is supported by our finding that inhibition of proteasomes, which blocks

Fig. 8. Effect of p53 AS-ODN on proliferation and DNA synthesis of various cell lines. HDFs (A), BE (B and E), SK-MEL-5 (C and F), SK-MEL-28 (H), BL-41 tsp53-2 cells (32°C and 37°C; D) and HL-60 (G) were treated with p53 AS-ODN, as described in the legends of Figs. 2 and 4, for 24 or 48–72 h. Mean values and SD of duplicates are shown. The experiments were repeated two to four times with similar results.

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Mdm-2 targeting of wt p53 (28, 29), decreased the IGF-IR activity in SK-MEL-5 cells.

Previous studies from our laboratory and other laboratories have shown that IGF-IR is crucial for the growth and survival of melanoma cells (17, 24, 32, 35–38). The incidence of p53 mutations in melanoma is very low, whereas p53 overexpression is common (39–42). A possible mechanism underlying loss of suppressor function of p53 in melanoma might be interaction between p53 and Mdm-2. Based on our present study, this may cause up-regulation of IGF-IR, which in turn may contribute to the malignant phenotype of melanoma.

We believe that the mechanisms mediating the regulatory effect of p53 on IGF-IR are complex. Therefore, further exploration of the involved molecular mechanisms for p53-dependent IGF-IR expression in malignant cells is needed. Another possibility that remains to be investigated is whether aberrant p53 interferes with regulatory steps at posttranscriptional levels.

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