Evidence for Nonautonomous Effect of \textit{p53} Tumor Suppressor in Carcinogenesis

Hippokratis Kiaris, Ioulia Chatzistamou, George Trimis, Matrisa Frangou-Plemmenou, Agatha Pafiti-Kondi, and Anastasios Kalofotis

\textsuperscript{1}Department of Biological Chemistry and \textsuperscript{2}Department of Pathology, Aretaieion Hospital, University of Athens Medical School, Athens, Greece

Abstract

Prostate, breast, and probably other epithelial tumors harbor inactivating mutations in the \textit{p53} tumor suppressor gene in the stromal cells, implying the nonautonomous action of \textit{p53} in carcinogenesis. We have tested this hypothesis by evaluating the tumorigenicity of MCF7 human breast cancer cells in severe combined immunodeficient mice that differ in their \textit{p53} status. Our results showed that, indeed, \textit{p53} ablation in the hosts reduced the latency for the development of MCF7 tumors. Furthermore, we show that heterozygous hosts frequently undergo loss of heterozygosity at the \textit{p53} locus in the tumor stroma tissue by mechanism that resembles the inactivation of \textit{p53} in primary tumors. To evaluate the impact of \textit{p53} ablation in the stromal fibroblasts, in tumorigenesis, tumors were reconstituted in mice bearing wild-type \textit{p53} alleles, by mixing MCF7 cells with fibroblasts isolated from mutant or wild-type \textit{p53} mice. Our results suggest that tumors containing \textit{p53}-deficient fibroblasts developed faster and were more aggressive than their counterparts with wild-type fibroblasts, although their neoplastic component, namely MCF7 mammary carcinoma cells, was identical in both cases. These data strongly support the notion for the operation of a nonautonomous mechanism for \textit{p53} action in primary tumors and provide a mechanistic association between \textit{p53} mutations in the stromal component of epithelial tumors and carcinogenesis.

Introduction

The \textit{p53} tumor suppressor gene plays an important role in the regulation of the apoptotic response of cells following exposure to genotoxic stress. Inactivating mutations at the \textit{p53} gene represent the most common genetic lesion of human primary tumors and have etiologically been associated with the onset of neoplasia (1). Whereas the implication of \textit{p53} in carcinogenesis is predominantly viewed as a cell-autonomous process, the recent identification of \textit{p53} mutations in the stromal component of primary breast and prostate tumors, in association with the role of stromal fibroblasts in tumor development, implies the operation of additional, nonautonomous mechanism(s) for the action of \textit{p53} gene in vivo (2–7). To experimentally test this hypothesis, we evaluated the growth properties of cancer cells in vivo, in mice that differ in their \textit{p53} status (8), using MCF7 human breast cancer cells as a model. Our results showed that, indeed, the genetic background of the stroma with respect to the status of \textit{p53} plays an important role in
the kinetic profile of tumorigenesis. Furthermore, tumor reconstitution experiments have shown that the fibroblastic component of tumors is sufficient to modulate both the latency of tumorigenesis as well as the morphology of the resulting tumors.

Materials and Methods

Mice. Severe combined immunodeficient (SCID) mice and \textit{p53}-deficient mice (2) were originally obtained by The Jackson Laboratory (Bar Harbor, ME). For the generation of SCID/SCID mice with the appropriate \textit{p53} status, male SCID/SCID mice were bred with homozygous \textit{p53}-null females. Subsequently, F1 double-heterozygous animals were bred to obtain in F2 SCID/SCID mice bearing wild-type or mutant copies of \textit{p53} in homozygosity or heterozygosity. Care of animals was in accordance with institutional guidelines.

Cell Culture, Xenograft Development, and Analysis. MCF7 mammary epithelial adenocarcinoma cells were originally obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM containing 10% fetal bovine serum. For xenograft development, 10\(^6\) MCF7 cells were injected s.c. in 3-week-old female SCID mice, of the genotype indicated for \textit{p53}, as described (9) and subsequently observed daily for tumor development. Allele-specific PCR assessing the \textit{p53} zygosity status of tumors was done in DNA extracted using standard phenol-chloroform extraction, and at amplification conditions suggested by the animal provider for genotyping with the modifications stated at the legend of the corresponding figure. The primers used were 5\’-TATACTCAGAGCCGGCCT-3\’ (1), 5\’-ACAGCGTGTTGGTACCTATAT\’-3\’ (2), and 5\’-TCTCTGTGCTTTACGGTATC-3\’ (3), which specifically amplified the wild-type (470 bp, 1 + 2) or the mutant (600 bp, 1 + 3) mouse \textit{p53} allele. Tumor reconstitution experiments were done as described above with the exception that \textit{p53}-wild-type or \textit{p53}-null mouse embryonic fibroblasts (MEF), isolated using standard methods, were mixed with the MCF7 cells at a ratio indicated before the inoculation into SCID mice. For histologic analyses, tumors were fixed in 10% formalin, paraffin embedded, and stained with H&E for light microscopy. Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) analysis was done by using the \textit{in situ} cell death detection kit (Roche, Basel, Switzerland) according to manufacturer's instructions. Immunohistochemistry for Ki-67 was done with a rabbit polyclonal antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA) by using the Ki67-DAB kit (ThermoShandon, Pittsburgh, PA) according to manufacturer's instructions. Before observation, a weak counterstain with hematoxylin was done. Images shown were obtained by Pro-Image Analysis Software (Media Cybernetics, Inc., Silver Spring, MD).

Results and Discussion

To obtain susceptible hosts for tumor transplantation studies, immuno-incompetent SCID/SCID mice were generated by selective breeding, bearing wild-type or mutant copies of \textit{p53} in homozygosity or heterozygosity. Animals were then inoculated with human MCF7 mammary cancer cells and the latency for the development of palpable tumors was recorded. As shown in Fig. 1A, tumorigenicity was considerably increased when MCF7...
cells were injected in p53-deficient animals compared with wild-type hosts, with heterozygous animals showing intermediate latency: Thirty-five days after cell injection, 90% of the p53-deficient animals had developed palpable tumors compared with ~ only 50% of their wild-type littermates. Thus, it seems that p53 deficiency in the host is sufficient to modulate the growth profile of cancer cells, such as MCF7 breast carcinoma cells in vivo.

Considering that loss of heterozygosity and subsequently reduction to homozygosity represents a common genetic alteration of p53 mutation-bearing tumors, we asked if such mechanism for p53 inactivation is also operative in the stromal component of the tumors bearing p53 mutations. In the present heterologous system in which the stromal, host-derived, component of the tumor is of mouse origin, whereas the cancer cells are of human origin, specific assessment of the p53 zygoisty status of the stromal cells in heterozygous animals is feasible by allele-specific PCR analysis. Consistent with the frequent presence of loss of heterozygosity of p53 in stromal cells of primary human tumors (3, 4), in six of eight tumors tested, the wild-type copy of p53 was deleted in the stromal cells (Fig. 1B). This finding may also explain the intermediate latency of tumorigenesis we observed.

Figure 1. A, tumorigenicity of MCF7 mammary carcinoma cells in p53+/+, p53+/−, and p53−/− animals. Three-week-old female SCID mice of the genotypes indicated where inoculated s.c. with MCF7 breast cancer cells as described (9) and observed daily for tumor development. Due to their increased mortality (14), three p53-null/SCID double-mutant animals died before they develop MCF7 xenografts and were excluded from the analysis. B, allele-specific PCR analysis for p53 in the stromal component of tumors grown in p53+/- animals. Tumors T1 and T2 exhibit loss of the wild-type (wt) p53 allele, whereas tumor T3 shows retention of heterozygosity, not with standing the fact that some allelic imbalance is apparent. PCRs were done for 26 cycles for the tail DNA (N1-N3, normal tissue) and for 35 cycles for the tumor DNA (T1-T3). Further increase in the cycle number of the tumor DNAs revealed the existence of wild-type p53 alleles, implying some heterogeneity regarding the cellular population subjected to loss of heterozygosity at p53. C, tumorigenicity of MCF7 mammary cancer cells mixed with MEFs bearing wild-type or p53-null alleles inoculated into SCID/SCID mice s.c. (n = 5 mice per group) at a ratio of 2 × 10⁶ MCF7 cells to 5 × 10⁵ MEFs. Mice were sacrificed 3 weeks after cell injection and tumors were dissected, formalin fixed, and paraffin embedded for histologic examination. The presence of p53-null MEFs considerably increased the tumorigenicity of the MCF7 cells compared with MEFs with wild-type p53. The difference in the latency between experiments shown in A and C is due to the fact that in the first case 10⁶ MCF7 cells/mouse were injected, whereas in C a ratio of 2 × 10⁵ MCF7 cells to 5 × 10⁵ MEFs/mouse was used.

Figure 2. Histology of MCF7 tumors reconstituted with p53-null (A) or wild-type (B, C) MEFs. Tumors containing p53-null MEFs were consistently characterized by low differentiation and stroma of high cellularity and reactivity (A). The genotype of the MEFs is shown in the upper right corner of each picture. Sections were stained with H&E using standard methods (magnification, ×20).
observed in the heterozygous p53 hosts, which is most likely due to a mechanism involving the stochastic inactivation of the wild-type p53 allele.

A formal argument against the specific role of the fibroblast could be that the contribution of the host’s genetic background in tumorigenesis of MCF7 cells can be due to cell types in the stroma other than the fibroblasts. To address this point, we have reconstituted MCF7 tumors in SCID/SCID mice bearing wild-type p53 alleles by mixing MCF7 cells with MEFs isolated from wild-type or p53-null animals and monitored the latency for development of palpable tumors. As shown in Fig. 1C, the presence of p53-null fibroblasts accelerated tumor formation compared with that of MCF7 cells mixed with wild-type fibroblasts. Thus, we conclude that the genetic background of fibroblasts is sufficient to affect the tumorigenicity of MCF7 cells.

Histologic examination indicated that the resulting tumors exhibited various differences depending on whether the MCF7 cells were mixed with wild-type or p53-null fibroblasts (Figs. 2 and 3). Tumors reconstituted with wild-type MEFs were in general well differentiated and in some instances exhibited typical ductal formation (Fig. 2B, arrows) showing mild stromal reaction (Figs. 2C and 3B and D), whereas tumors with p53-null MEFs were consistently of lower differentiation with more reactive stroma (Figs. 2A and 4A and C). Besides their difference in the degree of stromal reactivity, the stromal component from the tumors containing p53-null MEFs was characterized by higher cellularity than that of tumors with wild-type MEFs and the cancer cells exhibited higher pleomorphism and atypia (Fig. 3A versus B). Occasionally, clusters of apoptotic or necrotic cells were also apparent in the tumors with wild-type MEFs (Fig. 3D, arrows). Ki-67 staining and TUNEL assay, which stain preferentially proliferating and apoptotic cells, respectively, confirmed these findings and indicated that cell proliferation was in general slightly elevated in the tumors containing p53-null MEFs; on the other hand, tumors bearing wild-type p53 MEFs exhibit higher TUNEL positivity (Fig. 4). The latter is in agreement with the recent demonstration that under certain conditions, fibroblasts in the stroma may play a negative role in tumor development (10). The mechanistic basis regarding how p53 deficiency in the fibroblastic component of tumors affects tumorigenesis remains obscure. Both qualitative and quantitative changes in the stromal composition may account for these observations. It is
conceivable that p53 mutations in the fibroblasts result in bypassing cell cycle arrest, and, thus, as they increase in number they create a cellular milieu that supports the neoplastic growth of MCF7 cells more efficiently. Indeed, recent data showed that fibroblasts from tumor stroma have a distinct genetic profile and morphologic properties from those of normal tissue (11, 12). Alternatively, and considering previous findings showing that carcinoma-associated fibroblasts induce the malignant transformation of immortalized prostate epithelial cells whereas fibroblasts from normal epithelium do not, we may assume that p53 deficiency mimics, to some extent, the phenotype of the carcinoma-associated fibroblasts (2, 13). It has to be mentioned that by using an anti-CD34 antibody, which specifically stains the endothelial cells, we found that tumors of both genotypes were negative for endothelial cells, whereas neovascularization was limited to the periphery of the tumors (data not shown). Thus, differences in the tumor angiogenesis do not seem to be predominantly responsible for these findings at least during early stages of xenograft development. It is conceivable, however, that at subsequent stages of xenograft development, differences in the degree of tumor vascularization will be apparent between the two experimental groups.

Collectively, our results provide evidence for the operation of nonautonomous mechanism(s) by which p53 interferes with the tumorigenic process and attribute etiologic association between the previously reported p53 mutations in the stromal fibroblasts and carcinogenesis. Whether analogous, nonautonomous effects in tumorigenesis are elicited by other tumor suppressor genes bearing mutations in the stromal cells remains to be seen.

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References

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