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ESTABLISHMENT OF A HUMAN PANCREATIC ADENOCARCINOMA CELL LINE (PSN-1) WITH AMPLIFICATIONS OF BOTH c-*myc* AND ACTIVATED c-Ki-*ras* BY A POINT MUTATION

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Summary: A human pancreatic cancer cell line, PSN-1, was established from pancreatic adenocarcinoma tissue that had been stored for 1.5 years at -80°C without any special treatment. The stored tissues were first transplanted into nude mice, and from the xenograft, the PSN-1 cell line was established. The original primary tumor and two metastatic lymph nodes were previously found to have 50-fold amplification of c-*myc* and also 3- to 6-fold amplification of activated c-Ki-*ras* with a point mutation from GGT to CGT at codon 12. PSN-1 cells are unique in that amplifications of both c-*myc* and activated c-Ki-*ras* are present in the same degree as the original tumors. These cells were also found to contain increased amounts of c-*myc* and c-Ki-*ras* transcripts.

We reported previously that a primary lesion and two lymph nodes with metastasis showed 50-fold amplification of c-*myc* and 3- to 6-fold amplification of activated c-Ki-*ras* with a point mutation (GGT to CGT) at codon 12, resulting in substitution of arginine for glycine and acquisition of transforming activity (1). This was the first case that amplifications of both c-*myc* and activated c-Ki-*ras* could be developed *in vivo*. The changes of oncogenes in this case supported the hypothesis proposed previously that at least two classes of oncogenes are necessary for transformation of primary fibroblasts (2, 3).

Here we report the establishment of a unique cell line, PSN-1. The tissue of primary pancreatic adenocarcinoma was stored for 1.5 years at -80°C without any special precaution for preservation of the viability of the tumor cells, such as freezing the tissue gradually in the presence of dimethylsulfoxide or glycerol aseptically. This frozen tissue was first transplanted

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into nude mice, and from the xenograft, the PSN-1 cell line was established. The PSN-1 cells were examined for the presence of amplification of c-*myc* and activated c-Ki-*ras*, and results were compared with those of the original tumors. This is the first report showing establishment of a cell line in which amplifications of c-*myc* and activated c-Ki-*ras* occurred as the original tumor tissues. The amounts of c-*myc* and c-Ki-*ras* transcripts were also shown to be increased markedly in PSN-1 cells.

MATERIALS AND METHODS

Establishment of a xenograft and a cultured cell line

Surgical specimens of the primary tumor and metastatic lymph nodes, with diagnosis of poorly differentiated adenocarcinoma of the pancreas, were quickly frozen by pressing them between metal plates that had been kept in liquid nitrogen for 2 to 3 minutes. The frozen squashed tissues were then stored at -80°C. This is a routine procedure for preserving surgical specimens for analyses of their DNA and protein. DNAs extracted from parts of these tissues, were analyzed for the activation and amplification of oncogenes (1, 4). After storage for 1.5 years, the frozen tissue of the primary tumor was tried for establishing a cell line. Approximately 1.0 g of frozen tissue was pulverized by the homogenizer in liquid nitrogen. The resulted powder was immediately suspended in 1 ml of RPMI 1640 medium without serum, and 0.2 ml aliquots were injected subcutaneously into three nude mice (Balb nu/nu).

The xenografted tumor was cut into small pieces and transferred to culture flasks containing RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum. The cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 in air.

Southern and Northern blot hybridization

DNAs were extracted from the tissues or the cells and Southern blot hybridization analyses were performed as described previously (1, 5). RNAs were extracted by the hot-phenol method (6) and analysed by Northern blot hybridization (7). Briefly, 10 μg of total RNAs were subjected to electrophoresis on 1% formaldehyde-agarose gel. Then they were transferred to a nitrocellulose filter, and subjected to Southern blot hybridization.

Probes

The *Cla*I-*Eco*RI fragment of human c-*myc* (purchased from Oncol Inc.) and inserts from HiHi380 containing the *Sst*II-*Xba*I fragment of v-Ki-*ras* (8) and pK•XE2.0 (1) were used as probes. pK•XE2.0 is a recombinant pBR322 clone containing the 2.0 kbp *Xba*I-*Eco*RI intron fragment between exon I and exon II of c-Ki-*ras*. These probes were labeled with [α-^{32}P]dCTP by nick-translation. The specific activity of these probes was more than 1 x 10^8 cpm/μg.

Other cell lines

H69, N-231 (small cell lung carcinoma) (9) and HL60 (acute promyelocytic leukemia) (10) were cultured cell lines. These cells were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum at 37°C under a humidified atmosphere of 5% CO_2 in air.

RESULTS

Establishment of PSN-1

Two weeks after transplantation of a suspension of the primary pancreatic adenocarcinoma tissue that had been kept at -80°C for 1.5 years into nude mice, a tumor developed in one of the three mice at the site of injection. No

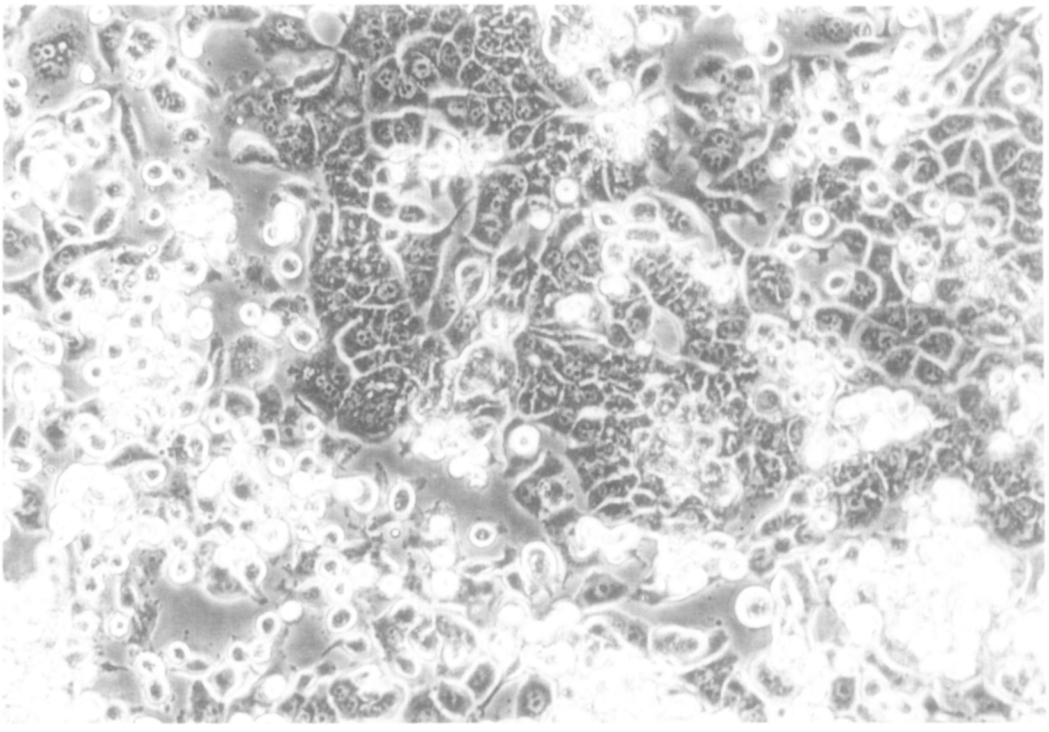

Fig. 1. Phase microscopic appearance of PSN-1 cells. x 200

tumor formation was observed in the other two mice even after 3 months. The tumor-bearing mouse was sacrificed and parts of the tumor were transplanted into other nude mice. The remainder of the xenograft was cut into pieces, and approximately 1.0×10^7 cells were transferred to culture medium. On day 3 of culture, cells started to grow and on day 5 they were distributed between two dishes. These cells have now been growing continuously for more than six months with a doubling time of 21 hours. Fig. 1 shows the phase microscopic appearance of PSN-1 cells. About one third of the cells were strongly attached to the substratum of the plastic flask, growing as monolayer cultures, and the others were weakly attached or grew in suspension. When the cells growing in suspension were transferred to fresh medium, approximately one third of the cells grew as monolayers. Conversely, when the monolayer cells were transferred to fresh medium after trypsinization, two thirds grew in suspension.

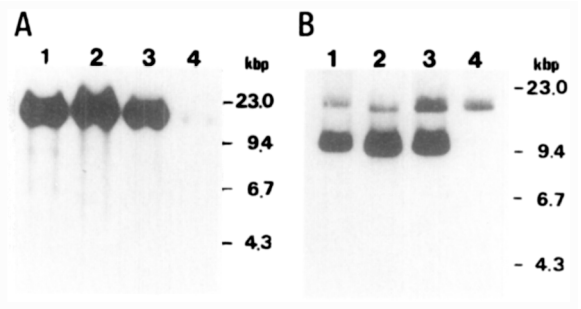

Fig. 2. Southern blot hybridization analyses of DNAs. Hybridization of *Eco*RI-digested DNAs with c-*myc* (A) and hybridization of *Sac*I-digested DNAs with pK·XE2.0 (B) were performed as described in the text. *Hin*dIII digested λDNA fragments were used as size markers. Lane 1, xenografted tumor; lane 2, PSN-1; lane 3, primary pancreatic tumor; lane 4, normal human spleen.

Southern blot hybridization

DNAs from xenografted tumor and PSN-1 cells were cleaved with restriction enzymes, and 10 μg samples of DNA digests was subjected to agarose gel electrophoresis and analyzed by Southern blot hybridization. *Eco*RI digested DNAs were hybridized with human c-*myc*. As shown in Fig. 2-A, xenografted tumor and PSN-1 cells showed 50-fold amplification of c-*myc*, like the original tumors.

As described previously (1), Southern blot hybridization of *Sac*I digests with pK·XE2.0 probe can detect a point mutation at codon 12 of c-Ki-*ras* by restriction fragment length polymorphism (1,11). As shown in Fig. 2-B, DNAs from the xenografted tumor and PSN-1 cells gave 9.4 kbp band, clearly demonstrating the existence of a point mutation (GGT to CGT) at codon 12 of c-Ki-*ras*. The intensity of the band was the same as that of the original tumors. PSN-1 cells also gave a 16 kbp fragment, indicating the presence of a normal c-Ki-*ras* allele.

Northern blot hybridization analyses

We examined whether these amplified oncogenes were reflected as increased mRNAs for c-*myc* and c-Ki-*ras*. Samples of 10 μg of total RNA of PSN-1 cells and three other cultured cells were compared by Northern blot hybridization analyses. As shown in Fig. 3, the transcripts of c-Ki-*ras* from PSN-1 cells were clearly increased, and the sizes of the transcripts were 5.6, 3.9, 2.6,

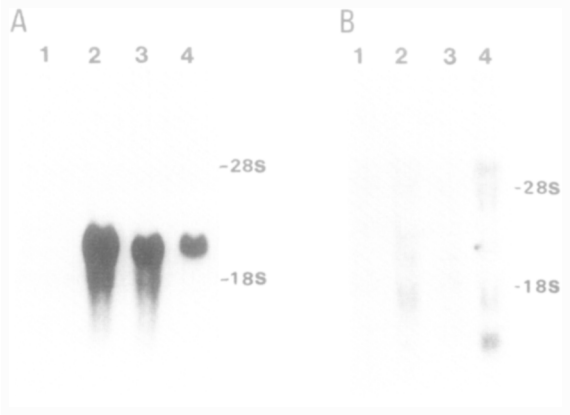

Fig. 3. Northern blot analyses of RNAs.
Samples of 10 μg of total RNAs were analyzed by Northern blot hybridization with c-*myc* (A) and with v-Ki-*ras* (B) as described in the text. Positions of ribosomal RNAs are indicated. Lane 1, H69; lane 2, N-231; lane 3, HL60; lane 4, PSN-1.

1.5 and 1.1 kb. Relative abundance of smaller sized transcripts was observed in PSN-1 cells. Moreover marked increase in the amount of c-*myc* mRNA was observed in PSN-1 cells, but the increase was less than in N-231 cells and HL60 cells, which show 15- and 20-fold amplification of c-*myc*, respectively (data not shown). The size of c-*myc* transcripts was 2.3 kb in PSN-1 cells as well as in N-231 and HL60 cells.

DISCUSSION

At least two classes of oncogenes are required for malignant conversion of normal cells (2,3). However, there are few reports of activation of two classes of oncogenes in tumors. Activation of two oncogenes was reported in HL60 cells, in which amplification of c-*myc* and activation of N-*ras* were observed and c-*myc* amplification was also observed in the original leukemic cells (12, 13). Amplification of c-*myc* and activation of c-Ki-*ras* in a xenograft of large cell carcinoma of the lung in nude mice has also been reported (14), but it was not clear whether these changes of the two oncogenes occurred during passage of the tumor in nude mice.

We previously reported the first demonstration of amplifications of both c-*myc* and c-Ki-*ras* with a point mutational activation at codon 12 in a primary pancreatic cancer and its metastatic tumors in lymph nodes (1). The point

mutation of c-Ki-*ras* involving change from GGT to CGT at codon 12 resulted in substitution of arginine for glycine and acquisition of transforming activity. We have now established a cell line, PSN-1, from the primary tumor of this pancreatic adenocarcinoma, and shown that it maintains amplifications of both c-*myc* and activated c-Ki-*ras* like the original tumors. By establishment of this unique cell line, we could clearly demonstrate the presence of a normal c-Ki-*ras* allele in tumor cells. The presence of normal allele was shown in the primary lesion and lymph node metastases *in vivo*, but we could not rule out the possibility that it was derived from the contaminating normal tissues or cells. We could also demonstrate that amplifications of c-*myc* and c-Ki-*ras* oncogenes were accompanied by increased expression of these two oncogenes. It should be noted that the same degree of amplifications of c-*myc* and activated c-Ki-*ras* existed stably in the primary and metastatic lesions *in vivo* and in the cultured cells *in vitro*. These findings suggest that the original tumor tissues were composed of homogeneous cells, showing the same degrees of amplification of c-*myc* and c-Ki-*ras*. These findings together with those showing increased expression of these oncogenes, suggest that these changes of oncogenes played a crucial role in the progression of the cancer.

The process for establishment of the PSN-1 cell line was unique in that it was achieved using frozen tissues that had been stored for analysis of DNA. Although, further studies are required to see whether the procedures used here have general applicability for establishment of cell lines from tissues frozen without preservatives, but it would be worth attempting to establish cell lines from frozen tissues, using the procedures described here.

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