Specific Detection of Metastasized Human Tumor Cells in Embryonic Chicks by the Polymerase Chain Reaction

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We have established a highly sensitive method for specific detection of metastasized human tumor cells in embryonic chicks using the polymerase chain reaction (PCR). The cells (HT-1080 or KMST-6) were inoculated into the chorioallantoic membrane vein of the chick embryo and DNA of each embryonic organ was extracted. Then, a human β -globin-related sequence (576 bp) in the DNA from the embryonic liver and lung was specifically amplified and detected by gel electrophoresis and by a specific oligonucleotide probe. The amplified fragments from the liver DNA samples increased gradually from 2 h to 7 days after HT-1080 inoculation. On the other hand, with inoculation of non-tumorigenic human embryonal fibroblast KMST-6 cells, the DNA from the embryonic liver 7 days after inoculation did not show the PCR-amplified product. This detection technique can contribute significantly to the precise detection of microscopic metastasis.

Key words: Metastasis — Chick embryo — Human fibrosarcoma HT-1080 — Human embryonal fibroblast KMST-6 — Polymerase chain reaction

There is no experimentally useful method for measurement of tumor growth in metastatic sites. Despite its clinical importance, the experimental restrictions imposed by this problem account for the limited study of chemosensitivity of metastases and also of analysis of the metastatic process. The naturally immunodeficient chick embryo has been used by us and many others to measure the metastatic properties of cells.¹⁻¹⁰ In those studies, however, the major disadvantage of using the chick embryo has been the time limitation imposed by the development of the embryo and its immune system. Because most tumor cells cannot produce macroscopic metastases in this system, the measurement of metastatic cells in chick embryo organs has been difficult. Recently, the polymerase chain reaction (PCR) technique has been developed for highly sensitive detection by amplifying a small segment of DNA flanked by oligonucleotide primers of known DNA sequences.¹¹ In this study, we could specifically detect DNA derived from human fibrosarcoma HT-1080, which metastasized in the chick embryonic liver and lung, by PCR using specific primers for human DNA and found that the technique was simpler, more sensitive, more specific, and more quantitative than the previously reported methods^{5,8} for the detection of the metastasized tumor cells.

Human fibrosarcoma HT-1080 cells, originally derived from a primary human acetabular bone tumor,¹² and human fibroblast KMST-6 cells, originally derived from a normal human diploid fibroblast strain (KMS-6) by treatment with ⁶⁰Co gamma ray irradiation,¹³ were used. Cells (HT-1080, KMST-6, or saline for control) were injected into chorioallantoic membrane (CAM) veins of 10-day chick embryos as described previously.¹⁴ Embryos were returned to humidified, 37°C chick embryo incubators for 7 days (or for the stated times for growth kinetics experiments). Embryonic livers and lungs were then dissected and DNA was extracted using a rapid DNA preparation method.¹⁵ We selected 576 bp of the β -globin gene region for amplification. We used Hu β -1 and Hu β -8 as the primers and Hu β -2 as the probe. Sequences of the primers for amplification and of the probe for detection are shown in Fig. 1. DNA from the embryonic organs was amplified by 25 cycles of PCR with the primers Hu β -1 and Hu β -8 and analyzed by agarose gel electrophoresis. Then, reaction products were hybridized with the probe Hu β -2, specific for the target DNA fragment.

First, the PCR amplification products from serial dilutions of HT-1080 DNA in normal embryonic liver DNA (total DNA amount, 1 μ g) were analyzed by Southern blotting. The results in Fig. 2 indicate that the PCR products amplified with the primers, Hu β -1 and Hu β -8, could be detected even at the concentration of 10^{-5} μ g of HT-1080 DNA (2×10^3 HT-1080 cells per embryonic liver tissue) but DNA from normal embryonic liver did not produce any PCR-amplified fragments under our

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- a Huβ-1: 5'-AGAGCCATCTATTGCTTACA-3'
 - b Huβ-8: 5'-TATGACATGAACTTAACCAT-3'
 - c Huβ-2: 5'-ACACAACTGTGTTCAGTACTAGC-3'

Fig. 1. Oligonucleotide primers and probe for PCR amplification and their locations in the human β -globin gene. (A) Sequences of oligonucleotide primers and probe used in PCR. (a) The primer Hu β -1 is complementary to the (–)-strand. (b) The primer Hu β -8 is complementary to the (+)-strand. (c) Hu β -2 was used as the probe to detect the amplified DNA fragment. (B) Location of the oligonucleotides. The amplified segment is 576 bp with Hu β -1 and Hu β -8. The downward-pointing arrow indicates the β -globin initiation codon and filled boxes indicate the β -globin encoding regions.

Fig. 2. Southern analysis of PCR amplification products from serial dilutions of HT-1080 genomic DNA in embryonic liver DNA. The result from undiluted normal embryonic liver DNA is shown as "chick liver." The PCR was done essentially as described by Saiki *et al.*¹¹⁾ We did the PCR with special precautions to prevent DNA contamination, as Kwok and Higuchi proposed.¹⁶⁾ The reaction mixture contained 1 μ g of genomic DNA, 1 $\times$ amplification buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin], each primer at 1 μ M, 200 μ M of each dNTP (dATP, dCTP, dGTP, TTP), and 2.5 units of polymerase in a total volume of 100 μ l. The samples were amplified by 25 cycles of PCR, each consisting of 2 min of denaturing at 94°C, 2 min of annealing at 55°C, and 2 min of extension at 72°C. After amplification, the DNA was concentrated by ethanol precipitation and all of the reaction mixture was electrophoresed on a gel of 1.5% agarose in TAE buffer [40 mM Tris-base, 5 mM sodium acetate, 1 mM EDTA, pH 7.6]. The gel was photographed, and the DNA was transferred to a nylon membrane filter and hybridized overnight to a ³²P end-labeled probe specific for the target DNA fragment at 43°C in a solution containing 6 $\times$ SSC, 0.1% SDS, 5 $\times$ Denhardt's solution,¹⁷⁾ denatured salmon sperm DNA (100 μ g/ml), and 10% dextran sulfate. The filter was then washed once in 2 $\times$ SSC-0.1% SDS at room temperature, twice for 15 min at 45°C, and once in 0.1 $\times$ SSC-0.1% SDS for 60 min at 45°C. The autoradiogram was exposed for 3–4 h with two intensifying screens at –80°C.

standard conditions; this region of the human β -globin genome and these primers are useful for specific amplification and detection of HT-1080 DNA. The same result was obtained in KMST-6 DNA (data not shown).

Second, the PCR-amplified products of DNA samples from embryonic organs after injection of 10³ to 10⁶ HT-1080 cells into the CAM vein were examined by Southern blot analysis (Fig. 3). DNA samples from embryonic liver inoculated with tumor cells produced amplified DNA fragments of the human β -globin gene. The amount of the PCR products was different depending on the inoculum tumor size. The inoculum of 1 $\times$ 10⁶ cells produced the most PCR products. Similar results were obtained for embryonic lung DNA samples.

Third, we examined the kinetics of the growth of HT-1080 and KMST-6 cells in embryonic liver to find whether the PCR-amplified fragments of the human β -globin gene, which were obtained from embryonic liver samples 7 days after tumor inoculation, came only from cells trapped in the liver following injection or from growing metastatic cells. The results are shown in Fig. 4. The amplified fragments from the liver DNA samples increased gradually from 2 h to 7 days after tumor

Fig. 3. Detection of metastasis in embryonic liver (A) and lung (B) following iv injection of HT-1080 cells. Numbers indicate the cell number injected into the CAM vein; two embryos received tumor cells at each inoculum size. Embryos were incubated for 7 days after tumor cell inoculation and their livers were dissected and weighed, and the DNA was extracted. DNA samples were amplified by 25 cycles of PCR and hybridized to the probe Hu β -2 as described in Fig. 2.

Fig. 4. Kinetics of growth of HT-1080 and KMST-6 cells in embryonic liver after iv injection (10^6 cells/embryo). Two groups of 10-day embryos received either HT-1080 (A) or KMST-6 cells (B). Three embryos from each group were killed at the stated times (2 h, 1, 3, 5, and 7 days) after cell inoculation. Embryonic livers were dissected and weighed, and the DNA was extracted. DNA samples were amplified by 25 cycles of PCR and hybridized to the probe Hu β -2 as described in Fig. 2.

inoculation. In the case of HT-1080 (inoculum size, 10^6 cells/embryo), the cell numbers per liver tissue estimated from the amount of the PCR fragment were 3.3×10^4 on

day 0 (2 h), 2.6×10^5 on day 1, 1.0×10^6 on day 3, 3.1×10^6 on day 5, and $>6.2 \times 10^6$ on day 7. On the other hand, in the case of KMST-6, DNA samples from embryonic liver from 2 h to day 3 produced the PCR-amplified fragments, but at a much lower level of the PCR products than HT-1080 cell injection. These products decreased by day 5 and were not detectable on day 7. The results indicate that HT-1080 cells were already trapped in the liver during the first 2 h after injection, and metastatic cells grew logarithmically during the 7 days, while non-tumorigenic KMST-6 cells were also trapped in the liver but they could not grow there. The PCR products obtained from liver DNA samples 7 days after tumor inoculation were from the growing metastatic cells, indicating a direct relationship between PCR product and experimental metastasis.

As was clear from these findings, the quantitative and specific detection of micrometastasis in chick embryo can be done using the PCR DNA-amplification technique. The chick embryo assay system may be useful not only for study of the cellular and molecular aspects of human metastasis but also for examination of agents inhibiting metastasis. The technique may also be useful for the quantitative study of metastasis in other *in-vivo* models such as assays using athymic nude mice.

This work was supported in part by Grants-in-Aid for Encouragement of Young Scientists (01790469) and Cancer Research (01010024) from the Ministry of Education, Science and Culture of Japan. We thank Dr. J. Tanaka of this Institute for providing KMST-6 cells.

(Received April 11, 1990/Accepted May 26, 1990)

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