In Vitro Irradiation Is Able to Cause RET Oncogene Rearrangement

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Abstract

Elevated risk of thyroid cancers among the atomic bomb survivors as compared to the nonexposed population suggests that some genetic events related to thyroid cancer must be caused by ionizing radiation. Accordingly, inducibility of RET oncogene rearrangements, i.e., the generation of the RET-PTC oncogene, specific for thyroid cancer, was investigated among human undifferentiated thyroid carcinoma cells (8505C), which do not have RET oncogene rearrangement, after 0, 10, 50, and 100 Gy of in vitro X-irradiation by means of reverse transcription polymerase chain reaction. After testing 10⁶ cells at each dose point, 3 independent samples obtained with 50 Gy of X-irradiation and 6 independent samples obtained with 100 Gy of X-irradiation showed a rearranged RET oncogene amplified band. No rearranged transcripts were obtained from cells irradiated with 0 or 10 Gy. All of the transcripts were sequenced and found to contain the D10S170 and RET sequence. Interestingly, two types of rearrangements were included in these transcripts: one is specific for thyroid cancer and the other, which contains a 150-base pair insert, is atypical, not usually seen in vivo. This inserted was found to be the exon of D10S170. Furthermore, in fibrosarcoma cells (HT1080), X-irradiation also induced RET oncogene rearrangements, which included the same two types of rearrangements observed in the X-irradiated thyroid cells (8505C). These results are in favor of the hypothesis that some radiation-induced thyroid cancers, including those among atomic bomb survivors, might have developed when a growth advantage was obtained through a specific form of RET oncogene rearrangement induced by radiation exposure.

Introduction

Epidemiological studies on the A-bomb survivors have shown elevated incidence of various kinds of solid tumors (1–3), in addition to leukemia. Although much information has been accumulated on the activation of oncogenes or the inactivation of tumor suppressor genes, there is no knowledge about the genetic events which are directly involved in cancers among A-bomb survivors. According to the current concept of multistep carcinogenesis (4), tumor development is considered to consist of multistep accumulation of adverse genetic and epigenetic events. Among these multiple genetic alterations, there must be specific genetic events uniquely associated with radiation carcinogenesis. For investigating these genetic events, thyroid cancer might serve as an interesting model because its incidence is elevated among A-bomb survivors (2, 3), and multistep genetic events are well studied in thyroid tumor development and progression (5–11).

Results to date suggest that point mutations in ras oncogenes detected in both adenoma and carcinoma seem to play a role in the initial stages of tumorigenesis (5, 6). In addition, rearrangements of the RET oncogene, often observed in PAC, is also suspected to be an early event during malignant conversion to thyroid cancer (7–10). As a late event, the tumor suppressor p53 gene has been found to be altered in undifferentiated thyroid cancer (11). Previous cytological studies have shown that various chromosome aberrations including deletions and rearrangements are observed among X-irradiated human cells (12–15). It follows, then, that because the tumor suppressor gene p53 often loses its function by deletions and the RET oncogene is activated by rearrangement, such events may be involved in radiation carcinogenesis of the thyroid. Among these genetic events, the RET oncogene is a good candidate for study on grounds that most thyroid cancers among A-bomb survivors are PACs, which are strongly associated with the RET oncogene. The RET protooncogene has been proposed to be a receptor tyrosine kinase. It is considered to be activated through gene rearrangement at a point just downstream of the transmembrane domain resulting in a chimeric gene between D10S170 and RET, both of which are located on the long arm of chromosome 10 (7). Such rearrangements can be easily detected by RT-PCR (8–10).

In this study, to identify whether the RET oncogene is actually associated with X-irradiation, inducibility of rearranged RET oncogene was investigated in a thyroid derived cell line after in vitro X-irradiation by means of RT-PCR.

Materials and Methods

Cell. The TPC-1 cell line, established from PAC of thyroid (16) and having a rearranged RET oncogene, was used as a positive control. Both the 8505C cell line (JCRB 0826), established in our laboratory from a primary undifferentiated thyroid carcinoma resected from a 78-year-old-female, and the HT1080 cell line, established from a fibrosarcoma (17), do not have a RET oncogene rearrangement and thus were used for the induction study.

X-irradiation. The X-ray generator (Shimadzu WSI-250S, Kyoto) was operated at 220 kVp, 8 mA with a 0.5 mm Al and 0.3 mm Cu filter; the dose rate was about 2.8 Gy/min. Actively growing 8505C cells were irradiated with 0, 10, 50, and 100 Gy in a 5-cm dish on ice and were subsequently incubated for 48 h under 5% CO₂ and 95% air at 37°C in RPMI-1640 supplemented with 10% fetal bovine serum, 1% l-glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin until RNA extraction.

Oligonucleotide Primers. PCR primers were synthesized on a DNA synthesizer (Applied Biosystems, Foster, CA). The sequences are: primer A, 5-GATAGACGAGGAGAAGCT-3; primer B, 5-GAGAGCCTCAAACGCT-3; primer C, 5-CAGAAACAAGATTCTGCTCAG-3; primer D, 5-TAGAGTTTTTCAAGAACCAAG-3; primer E, 5-GTGACCCATCTG-3; primer F, 5-GTGACCCATCTG-3. The approximate location of primers A–D, based on published sequence information for the typical forms of RET oncogene rearrangements (8). Primers E and F, based on published sequence information of c-BCR, were used for quantification of extracted RNA (18).

RNA Isolation and Amplification. Approximately 10⁶ cells irradiated at doses of 0, 10, 50, and 100 Gy were collected and lysed in a 4 µm guanidinium solution after being washed with phosphate-buffered saline. This was repeated over 10 times, and each of the extracts was pooled separately to end up with RNA from effectively 1 x 10⁸ cells/dose point. The RNA was then pelleted through a CsCl step gradient and ~100 µg total RNA was extracted from 10⁶ cells. Ten % of the extracted total RNA (~10 µg) was screened by RT-PCR followed by nested second round PCR. For optimization of the first round PCR conditions, approximately 10 µg of RNA were divided into 3-µg aliquots.
To exclude the possibility of degradation of extracted RNA, c-BCR was amplified from 250 ng of RNA by RT-PCR with primers E and F under 29 cycles of the previously described thermal conditions.

**Direct Sequencing.** Amplified PCR products were purified by electrophoresis with low melting agarose gel (3%). The purified products were sequenced directly using primers B and C, one of which is labeled at its 5' end with [γ-32P] ATP by T4 nucleotide kinase.

The direct sequencing was performed with double stranded DNA cycle sequencing system reagents (Bethesda Research Laboratories), using the modified dideoxy chain termination method of Sanger et al. (19).

**Results and Discussion**

The risk of thyroid cancer is elevated among A-bomb survivors and the main cellular target for ionizing radiation is thought to be DNA. However, the specific genes involved in thyroid tumor development and progression cannot be identified without the appropriate assays. This study demonstrated that the rearranged and consequently activated RET oncogene, a possible target gene, can be detected by a highly specific and sensitive RT-PCR assay. To determine the sensitivity of the RT-PCR assay, dilution experiments were carried out (Fig. 2) using TPC-1 cells bearing the RET oncogene rearrangement. TPC-1 cells were mixed with OKI B cells, an Epstein-Barr Virus transformed B cell line, in ratios varying from 1:10 to 1:105 (a total of 10^7 cells). After RNA was extracted from the mixtures, it was subjected to RT-PCR. A 117-base pair product could be detected from the TPC-1 cells even when mixed with B cells at a ratio of 1:10^5. It was confirmed that rearranged RET oncogenes were undetectable among untreated 8505C and HT 1080 cell lines. With this assay the inducibility of RET oncogene rearrangement among 8505C cells by X-irradiation was investigated, as shown in Fig. 3. No transcripts of rearranged RET oncogenes were detectable in the 1 x 10^8 cells X-irradiated with doses of 0 and 10 Gy. Three and 6 samples showed an amplified band corresponding to a rearranged RET oncogene from 1 x 10^8 cells X-irradiated with 50 and 100 Gy, respectively. Each lane in Fig. 3 represents the results from ~10^7 cells. Because RET oncogene rearrangements were induced at a frequency lower than 10^-6 with 0 or 10 Gy and at least 0.3 x 10^-7 with 50 Gy and 0.6 x 10^-7 with 100 Gy, a dose-response seems to exist. But further investigation is needed to discuss this point in more detail.
Fig. 3. The inducibility of RET oncogene rearrangement by X-irradiation. Three positive bands were identified in C (50 Gy) and six positive bands were identified in D (100 Gy). No products could be identified in A (0 Gy) and B (10 Gy). These positive bands, amplified from separately extracted RNA, originated from different cells. For confirmation of sufficient RNA extraction, c-BCR was amplified from RNA. Side lanes, φX174 HaeIII digested DNA markers.

All of the transcripts were sequenced and confirmed to contain the 10S170 and RET sequence. In addition it was found that two types of rearrangements were included in these X-irradiation induced transcripts. One is the typical rearrangement commonly observed in thyroid cancer (7–10) and the other is an atypical one with an unknown 150-base pair insertion at the junction site. A sequencing autoradiogram of the junction site from two types of transcripts is shown in Fig. 4. Although there is no information on the downstream sequence from the junction site of the D10S170 gene, it is suspected that this unknown sequence is composed of the D10S170 exons, since it is observed repeatedly among the fusion genes. To test this idea, RT-PCR analysis was performed using primer A and a newly synthesized 3' primer (5' -TGAATTCTTCTTCCTGCTCAG-3') complementary to this unknown insertion sequence. It became clear that this unknown insertion sequence originated from the adjacent exon of D10S170, just downstream of the typically seen junction site because the expected size products (114 base pairs) were amplified using these primers on RNAs of nonirradiated 8505C and HT1080 cell lines (data not shown). The insertion sequence is devoid of a stop codon and is 150 base pairs long resulting in the maintenance of the correct reading frame. The fact that the atypical transcript has not been found in vivo, even if the reading frame of the RET oncogene is correctly maintained, suggests that the region of importance for activity of tyrosine kinase is located upstream of the junction site, in a similar fashion to the NH₂-terminal domain of the c-ABL tyrosine kinase (20).

To date, RET oncogene activation by rearrangement has been found only in thyroid tumors (7–10). If this genetic event is truly seen only in thyroid tumor (9), the question arises whether the rearrangement itself or an acquired growth advantage is unique to the thyroid. In order to resolve this question, we examined the uniqueness of the RET oncogene rearrangements to the thyroid by using a thyroid cancer derived cell line, 8505C. After successful induction of rearrangement in the thyroid derived cell line, a sarcoma cell line, HT1080, was tested. RET oncogene rearrangements, including the same two types observed in the irradiated thyroid derived cells (8505C), were also induced by 100 Gy of X-irradiation in 1 × 10⁶ HT1080 cells (data not shown). The results suggest that induction of RET oncogene rearrangement is not unique to thyroid but that the acquired growth advantage is. Further investigation is needed in order to clarify this point.

When a rare mutant is investigated among a large population, a highly specific, sensitive, and convenient assay like nested PCR is imperative. However, to accomplish this kind of highly sensitive assay without contamination, great care must be taken. The results of the atypical transcripts are definitely not due to contamination because this is the first time these amplicons have been studied at this institute. Furthermore, to convince ourselves that the typical transcripts detected were not amplified from previous PCR products, we repeated this assay for positive cases with and without reverse transcriptase in duplicate. Samples with and without reverse transcriptase were repeatedly positive and negative, respectively, for the amplicons. These results exclude the possibility of amplified DNA contamination.
In this study, different types of rearranged RET, including one typically seen and another never seen in thyroid cancer, were induced by in vitro X-irradiation. The results suggest that different types of rearranged RET may be induced after radiation exposure in vivo; however, only the specific form may be positively selected exclusively through the acquired growth advantage resulting in development of thyroid cancer.

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