Formation of carcinogenic chromosomal rearrangements in human thyroid cells after induction of double-strand DNA breaks by restriction endonucleases

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Abstract

Ionizing radiation (IR) exposure increases the risk of thyroid cancer and other cancer types. Chromosomal rearrangements, such as RET/PTC, are characteristic features of radiation-associated thyroid cancer and can be induced by radiation in vitro. IR causes double-strand breaks (DSBs), suggesting that such damage leads to RET/PTC, but the rearrangement mechanism has not been established. To study the mechanism, we explored the possibility of inducing RET/PTC by electroporation of restriction endonucleases (REs) into HTori-3 human thyroid cells. We used five REs, which induced DSB in a dose-dependent manner similar to that seen with IR. Although all but one RE caused DSB in one or more of the three genes involved in RET/PTC, rearrangement was detected only in cells electroporated with either PvuII (25 and 100 U) or StuI (100 and 250 U). The predominant rearrangement type was RET/PTC3, which is characteristic of human thyroid cancer arising early after Chernobyl-related radioactive iodine exposure. Both enzymes that produced RET/PTC had restriction sites only in one of the two fusion partner genes. Moreover, the two enzymes that produced RET/PTC had restriction sites present in clusters, which was not the case for RE that failed to induce RET/PTC. In summary, we establish a model of DSB induction by RE and report for the first time the formation of carcinogenic chromosomal rearrangements, predominantly RET/PTC3, as a result of DSB produced by RE. Our data also raise a possibility that RET/PTC rearrangement can be initiated by a complex DSB that is induced in one of the fusion partner genes.

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Introduction

Ionizing radiation (IR) is a well-known carcinogen linked to a variety of human cancers. One of those is thyroid cancer, which has a well-established link to the exposure to external γ- and X-ray radiation (Schneider 1990, Ron et al. 1995, Schlumberger et al. 1999) and internal exposure to ingested I-131 (Tuttle & Becker 2000, Cardis et al. 2005a). The risk of thyroid cancer has a linear dose response for doses 0.1–2 Gy, with flattening of the dose–response curve at higher doses (Ron et al. 1995, Cardis et al. 2005a, b).

Over the last decade, strong evidence has been accumulated supporting the central role of chromosomal rearrangements in carcinogenesis initiated by IR (Ciampi et al. 2005, Gandhi et al. 2010). A prototypic example of such radiation-induced rearrangement is a RET/PTC rearrangement, which is highly prevalent in thyroid cancer in individuals exposed to IR (Bounacer et al. 1997, Rabes et al. 2000, Collins et al. 2002) and can be induced in thyroid cells by IR in vitro (Ito et al. 1993, Mizuno et al. 2000, Caudill et al. 2005). The two most common rearrangement types are RET/PTC1 and
RET/PTC3, in which RET is fused to either CCDC6 (H4) or the NCOA4 (ELE1) gene (Grieco et al. 1990, Santoro et al. 1994). RET/PTC1 is more common after γ-radiation, whereas RET/PTC3 was the predominant type in patients exposed to I-131 after Chernobyl who developed thyroid cancer <10 years after exposure (Bounacer et al. 1997, Nikiforov et al. 1997, Rabes et al. 2000). In vitro, the formation of RET/PTC can be found in thyroid cells exposed to external γ- and X-ray radiation (Ito et al. 1993, Mizuno et al. 2000, Caudill et al. 2005). The findings in the human populations and in experimental models provide strong evidence for the direct role of radiation in generating RET/PTC. Understanding of the mechanisms of formation of this carcinogenic rearrangement can therefore provide important insights into the mechanisms of radiation carcinogenesis in human thyroid cells and in other cell types.

IR is known to induce various types of DNA damage, of which double-strand breaks (DSBs) are considered to be a crucial primary lesion for a variety of biological end points, including chromosomal aberrations and cell transformation (Bryant & Riches 1989, Winegar et al. 1992). However, how exactly radiogenic DSBs lead to chromosomal aberrations remain unclear. Three basic theories have been proposed. The Breakage-and-Reunion theory postulates that chromosomal aberrations arise mainly as a result of rejoining of two DSBs located closely in space and time (two-hit event) (Savage 1998, Hlatky et al. 2002). Presumably, most rejoining events occur via nonhomologous end joining (Yates & Morgan 1993, Rothkamm et al. 2001). An alternative, one-hit mechanism is suggested by the Molecular theory, which postulates that one radiation-induced DSB is sufficient to initiate an exchange that occurs with an undamaged DNA molecule (Chadwick & Leenhouts 1978, Goodhead et al. 1993). The plausible mechanism for such a series of events is homologous recombination initiated by one DSB. The Exchange theory suggests that the initiation lesions are not DNA breaks induced by radiation but rather ‘unstable lesions’ that do not disrupt the continuity of chromosomes but can initiate exchange between two lesions (Revell 1975). Although the Breakage-and-Reunion theory remains most widely accepted, none of the three theories can adequately explain all available experimental data on the dose–effect relationship and complexity of radiation-induced aberrations (Edwards 2002). Moreover, these theories are based on the assumption that primary DNA lesions are directly induced by radiation. However, additional evidence exists that radiation can lead to chromosomal exchanges by an indirect mechanism, i.e. mediated by radiation-induced genomic instability. This possibility is supported by studies showing the occurrence of new chromosomal aberrations in subsequent generations of a cell exposed to radiation (Huang et al. 2003, Little 2003), and by a bystander effect, where aberrations are found in cells plated close to, but not in, the field of irradiation or partial irradiation of a cell cytoplasm (Ludwików et al. 2002, Morgan et al. 2002, Little et al. 2003).

In human tumors, the breakpoints of RET/PTC rearrangements typically reside within a 1.8 kb intron 11 of RET and in intronic sequences in CCDC6 (intron 7, 1.7 kb) and CCDC6 (intron 1, 53.4 kb), allowing for undisturbed splicing and expression of genetic chimeras. Analysis of genomic breakpoints in RET/PTC3 rearrangements in post-Chernobyl thyroid tumors showed random distribution of the breakpoints within the introns, presence of common regions of microhomology composed of three to five nucleotides at the breaks, and minimal modification of sequences at the breakpoints (Bongarzone et al. 1997, Nikiforov et al. 1999, Klugbauer et al. 2001). We have previously reported a dose-dependent generation of RET/PTC rearrangements by γ-radiation in thyroid HTori-3 cells, which are immortalized, partially transformed cells that preserved features of thyroid differentiation (Caudill et al. 2005).

IR breaks DNA at random location, which precludes investigation of the role of any particular break in the mechanism of RET/PTC formation. By contrast, restriction endonucleases (REs), which can be introduced into mammalian cells by electroporation, cause DSBs at defined chromosomal locations (Bryant 1988, Ager et al. 1991, Obe et al. 1992). REs have been widely used to study the biological consequences of DSBs in the genome, which include the generation of chromosomal rearrangements, among other effects. A linear dose response has been reported in studies on the frequency of chromosomal aberrations induced by restriction enzymes introduced into mammalian cells (Bryant 1984, Winegar et al. 1989, Yates & Morgan 1993).

In this study, we compared the characteristics of DSBs induced by IR and RE in thyroid cells and used RE induction of DSBs to study the generation of RET/PTC rearrangements in thyroid cells.

Materials and methods
Cell line and culture conditions
HTori-3 cells, which are normal human thyroid cells transfected with an origin-defective SV40 genome (Lemoine et al. 1989), were purchased from the
European Tissue Culture Collection (Salisbury, Wiltshire, UK). They are partially transformed but retain a differentiated phenotype as indicated by the ability to accumulate iodide and produce thyroglobulin (Tg). We have previously shown that our HTori-3 cells express both sodium/iodide symporter and Tg (Caudill et al. 2005). Primary cultures of human thyroid epithelial cells were established from surgically removed tissues as described previously (Nikiforova et al. 2000).

Cell irradiation

Human thyroid primary cells (2×10^3) or HTori-3 cells were plated in a 35 mm plate with coverslip and 16 h later exposed to a single dose of γ-irradiation from cesium-137 (Gamma Cell 40 irradiator) at a dose rate of 0.58 Gy/min. Cells were exposed to 0.1, 0.5, 1, and 5 Gy γ-irradiation and incubated at 37°C for different periods, 1, 2, 4, 6, 24, 48, and 72 h, and subjected to γH2AX (H2AFX) immunofluorescence.

Electroporation of restriction enzymes and detection of RET/PTC rearrangements

PvuII, StuI, EcoRV, ScaI, and NruI RE (New England Bio Labs, Ipswick, MA, USA) were electroporated into human thyroid primary cells or HTori-3 cells by a previously described method (Carney & Morgan 1999). Briefly, 2×10^6 cells in 800 μl PBS were electroporated with different amounts of enzymes at 50 μC charge (400 V and 125 μF) using Gene Pulser Xcell Electroporator (Bio-Rad). For the detection of RET/PTC, in each experiment, the electroporated cells were seeded in 30 T25 flasks (6.6×10^4 cells per flask). To sustain continuous growth, cells were transferred to T75 flasks on day 5 post-electroporation and harvested on day 9. RNA was extracted from each flask using a Trizol reagent (Invitrogen) and mRNA was purified using Oligotex mRNA mini kit (Qiagen). RT-PCR with primers specific for RET/PTC1 and RET/PTC3 was performed and RET/PTC detection was achieved by Southern blot hybridization of the PCR products with 32P-labeled oligonucleotide probes specific for each rearrangement type as described previously (Caudill et al. 2005). Evidence of RET/PTC rearrangement in the cells from a given flask was scored as one RET/PTC event.

Electroporation efficacy for all studied enzymes was assayed by γH2AX immunofluorescence after 4 h post-electroporation. Cell survival after electroporation of each RE was evaluated 24 h post-electroporation by trypan blue exclusion.

γH2AX immunofluorescence

γH2AX immunofluorescence was used to confirm DSB formation after irradiation and RE electroporation of human thyroid cells as well as for DSB quantitation. Cells cultured on coverslips were fixed with 4% formaldehyde for 10 min followed by the permeabilization with 0.5% Triton X-100 on ice for 5 min. After washing with PBS, cells were stained with 100 μl staining solution (20 mM Tris, pH 7.6, 137 mM NaCl, 10% skimmed milk, and 0.5% Tween-20) containing antiphosphorylated histone H2AX primary antibody (Upstate Biotechnology, Buffalo, NY, USA) at 1:1000 dilution for 2 h at 37°C. Coverslips were washed with PBS twice and incubated with Alexa488-labeled antimouse IgG (Molecular Probes, Eugene, OR, USA) in staining solution (1:200) for 1 h at 37°C. After washing with PBS, samples were counterstained with DAPI (Suzuki et al. 2006). In order to quantify the number of DSBs, γH2AX foci were counted in individual nucleus scanned using a Leica SPS TCS 4D confocal laser scanning fluorescence microscope with a 63×, 1.4 N.A. oil PlanApo objective. Twenty nuclei were scanned for each condition and the digital images were reconstructed using Volocity software (Perkin Elmer, Waltham, MA, USA). Image stacks were subjected to uniform 90% intensity thresholding along with separation of touching objects to demarcate the foci signals. This technique allowed for automatic quantification of γH2AX foci within the nuclear volume.

Fluorescent in situ hybridization

Touch preparations of primary thyrocytes and HTori-3 cells grown in chamber slides were subjected to 3D fixation in 4% paraformaldehyde followed by repeatable freeze–thaw cycles in liquid nitrogen (Cremer et al. 2008). RET, NCOA4, and CCDC6 probes were labeled by nick translation, hybridized, and analyzed as described previously (Gandhi et al. 2006).

Statistical analysis

The two-tailed t-test for independent samples was used to compare the number of γH2AX foci induced by irradiation and RE, distances between specific probe pairs, and rates of RET/PTC rearrangement formation in different experiments. The difference was considered statistically significant when P was <0.05.

Results

Restriction enzymes simulate IR-induced DSBs in human thyroid cells

First, we evaluated the ability of the RE PvuII to induce DSBs at known chromosomal sites in primary cultures
of cells (PCCs) established from normal human thyroid tissue. DSB formation was monitored by immunostaining for γH2AX foci, which form at DSB sites (Rogakou et al. 1998, Sedelnikova et al. 2002). We observed that the number of γH2AX foci generated by PvuII was increasing with the increased dose of enzyme (Fig. 1). The dose response was close to linear when the cells were electroporated in the buffer containing between 5 and 100 U PvuII. Higher doses of PvuII (250 and 500 U) produced very large regions of γH2AX staining, suggesting that foci had conglomerated. The foci induced by PvuII were diffusely distributed within the nuclear volume, which is similar to what is seen in cells treated with IR. However, in contrast to γ-radiation, which induced foci in 100% of cells, PvuI induced foci in 70–89% of cells. Nevertheless, when only cells that had an increased number of γH2AX foci were scored, the number of foci induced by 25 and 100 U of electroporated PvuII were comparable with the number of foci induced by 1 and 3 Gy of IR, respectively, which is the dose range known to produce RET/PTC rearrangements in thyroid cells in vitro (Caudill et al. 2005) and induce thyroid cancer in human populations (Ron et al. 1995, Cardis et al. 2005a, b). These data provided evidence that DSB can be induced in human cells by RE in a dose-dependent manner and in quantities and distribution similar to DSB induced by IR doses known to cause carcinogenic chromosomal rearrangements.

Then, we investigated whether the kinetics of induction and repair of DSBs induced by PvuII and by γ-radiation is similar between PCC cells and immortalized HTori-3 thyroid cells. After exposure to γ-radiation, the fraction of cells with γH2AX foci in both types of cells was highest at the earliest time point examined (15 min post-irradiation) and declined with similar kinetics (Fig. 2A). In both cell types, the fraction of cells with induced foci declined more

Figure 1 Comparison of DSBs induced by γ-radiation and PvuII in primary cultured thyroid cells evaluated based on γH2AX foci formation. (A) γH2AX foci distribution in cells. (B) Correlation between the number of induced γH2AX foci and radiation dose and dose of PvuII.
slowly in cells treated with 5 Gy compared with 1 Gy. Similar results were obtained in cells electroporated in the presence of low (25 U) and high (250 U) dose of PvuII, except that it took longer for cells with foci to reach its maximum and return to baseline (Fig. 2B). In both cell types, the fraction of cells with induced γH2AX foci was higher in cells that were electroporated in the presence of 250 U compared with 25 U PvuII, and this fraction declined more slowly (Fig. 2B). The slower kinetics of focus formation and dissolution after RE treatment were not surprising given the requirement for transport of the enzyme into the nucleus and the known persistence of endonuclease activity for up to 12 h after electroporation (Costa & Bryant 1991), in contrast to the virtually immediate one-time induction of DSBs by IR. These results indicated that both types of cells repaired induced DSBs with similar kinetics. In addition, using 3D fluorescent in situ hybridization (FISH) analysis, we determined that HTori-3 cells, known to have three copies of intact chromosome 10, preserve spatial proximity between the RET, NCOA4, and CCDC6 gene loci which has been previously observed in normal human thyroid cells (Nikiforova et al. 2000, Gandhi et al. 2006; Supplementary Figure 1, see section on supplementary data given at the end of this article). These findings indicate that induction of DSBs by electroporation of RE into a stable cell line (HTori-3 cells) could be used as a model to study the mechanisms of RET/PTC rearrangement that arise in human thyroid cells after exposure to IR. Moreover, HTori-3 cells provide a source of large quantity of stably dividing human thyroid cells that are required for such experiments and which cannot be obtained by culturing normal human thyroid cells.

**REs induced RET/PTC rearrangements with predominance of RET/PTC3 type**

The ability of restriction enzymes to induce RET/PTC rearrangements in thyroid cells was tested using HTori-3 cells and five blunt-end cutting REs: PvuII, EcoRV, NruI, ScaI, and StuI. The enzymes chosen induced DSBs in >60% of cells at a dose that allowed more than 70% of the electroporated cells to survive. The panel of enzymes was also chosen in order to test the validity of the three different theoretical models of radiation-induced rearrangement. Consequently, the enzymes used differed with respect to their capacity to cleave the sequences that become joined in RET/PTC1 and RET/PTC3 rearrangements. In RET/PTC1, intron 11 of RET recombines with intron 1 of CCDC6. In RET/PTC3, intron 11 of RET recombines with intron 7 of NCOA4. All three pertinent introns have ScaI cleavage sites, but intron 7 of NCOA4 lacks sites for PvuII and StuI, and intron 1 of CCDC6 is the only intron that has an EcoRV site (Table 1 and Fig. 3). None of the three introns have an NruI site. Therefore, if two breaks were needed (Breakage-and-Reunion theory), then ScaI would be expected to induce both RET/PTC1 and RET/PTC3 because there are sites for

**Table 1** Number and characteristics of restriction enzyme digestion sites in genes participating in RET/PTC rearrangements

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>RET intron 11, 1.8 kb</th>
<th>NCOA4 intron 7, 1.7 kb</th>
<th>CCDC6 intron 1, 53.4 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScaI</td>
<td>2 (332 bp)</td>
<td>2 (1274 bp)</td>
<td>9 (397 bp)</td>
</tr>
<tr>
<td>PvuII</td>
<td>3 (24 bp)</td>
<td>0</td>
<td>22 (327 bp)</td>
</tr>
<tr>
<td>StuI</td>
<td>4 (65 bp)</td>
<td>0</td>
<td>10 (551 bp)</td>
</tr>
<tr>
<td>EcoRV</td>
<td>0</td>
<td>0</td>
<td>4 (3271 bp)</td>
</tr>
<tr>
<td>NruI</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Distance between closest restriction sites is shown in brackets.*
this enzyme in all three of the pertinent genes. Similarly, PvuII and StuI would induce RET/PTC1 but not RET/PTC3, and EcoRV and NruI would not induce RET/PTC at all. However, if only one break were needed (the Molecular theory), then only NruI would be expected to fail to induce RET/PTC. The Exchange theory, which postulates the formation of initiation lesions that are not DSB, would seem to be not applicable to genomes damaged by restriction enzymes. Nevertheless, if NruI were to induce RET/PTC, such a result would support the hypothesis of a mechanism that does not directly involve one or more DSBs in the DNA sequences that recombine.

Induction of RET/PTC rearrangements was studied by RT-PCR using the approach we previously used to study the generation of this rearrangement by γ-radiation (Fig. 4; Caudill et al. 2005). RET/PTC rearrangements were detected after electroporation with either PvuII (25 and 100 U) or StuI (100 and 250 U) (Table 2). Each enzyme produced both rearrangement types, although RET/PTC3 was by far the most common type induced by PvuII. The rate of RET/PTC1 induction by both enzymes was 0.12–0.17 per 10^6 cells, whereas the rate of RET/PTC3 formation was 0.15–0.34 per 10^6 cells by StuI and 2.19–2.92 by PvuII. The other three restriction enzymes tested did not induce detectable RET/PTC rearrangement. Doses of PvuII and StuI that produced RET/PTC rearrangements induced between 40 and 200 DSBs per cell, as estimated based on the number of γH2AX foci. This amount of DNA damage is in the range induced by 1–5 Gy of IR (Sedelnikova et al. 2002).

**Induction of RET/PTC rearrangements correlated with clustering of RE cleavage sites in one of the fusion partners**

RET/PTC was found in cells treated with either StuI or PvuII, indicating that enzymatic DNA cleavage can cause these types of rearrangements. However, RET/PTC3 rearrangements greatly predominated in cells electroporated with PvuII, despite the fact that one of the RET/PTC3 fusion partners, the PTC3 intron 7, contains no PvuII sites (Table 1 and Fig. 3). The presence of wild-type sequences of RET intron 11 and NCOA4 intron 7 in HTori-3 cells was confirmed by nucleotide sequencing, and PCR products of these intronic sequences showed patterns of digestion expected based on their restriction maps (Supplementary Figure 2, see section on supplementary data given at the end of this article). On the other hand, induction of DSB in one gene was not always sufficient to produce RET/PTC because it was not generated by either EcoRV or Scal. These findings indicate that induction of RET/PTC was dependent on some other features of DNA damage induced by specific RE. Indeed, a unique feature of PvuII and StuI is that their sites are clustered in RET intron 11. Specifically, the intron contains two PvuII sites only 24 bp apart and
two StuI sites separated by 65 bp (Table 1). By contrast, relevant restriction sites of EcoRV and Scal are 300 bp or more apart.

Discussion

The results of this study demonstrate that carcinogenic chromosomal rearrangements can be generated in human thyroid cells as a result of DSBs caused by restriction enzymes. The predominant type of the fusion was RET/PTC3, which is characteristic of human thyroid cancer arising after exposure to I-131. Moreover, our results raise a possibility that RET/PTC can be formed in human thyroid cells as a result of a complex DSB produced in one of the genes participating in the fusion.

The preferential formation of RET/PTC3 after electroporation of RE found in this study was unexpected because RET/PTC1 is a predominant type of the rearrangement in most human populations and in experimental exposures to IR (Bounacer et al. 1997, Collins et al. 2002, Hamatani et al. 2008). The notable exception is a population of individuals exposed predominantly to radioiodines as a result of the Chernobyl accident who developed thyroid cancer predominantly to radioiodines as a result of the Chernobyl accident (Bounacer et al. 1997, Thomas et al. 2000, 2002). The I-131 isotope, which emits both γ-radiation and β-particles, has an even higher potential for producing complex DNA damage. The charged particles have more complex track structure and denser energy deposition than γ-radiation, with higher chance of inducing DSBs and clustered DNA damage (Goodhead 1994, Hada & Georgakilas 2008). In thyroid cells, I-131 induces DSBs detectable as γH2AX or 53BP1 foci (Hersman et al. 2011), as well as clustered DNA damage detected by Comet assay (Grzesiuk et al. 2006). Taken together, these data and results of our study raise at least a theoretical possibility that complex DSB in one gene is sufficient to initiate the generation of RET/PTC rearrangement in thyroid cells. This would support a one-hit mechanism postulated by the Molecular theory (Chadwick & Leenhouts 1978, Goodhead et al. 1993).

Alternately, it remains possible that the formation of RET/PTC3 occurred via two breaks, the first being formed by PvuII cutting at its canonical recognition...
sites and the second being formed in the NCOA4 gene by nonspecific cleavage. In vitro, under artificial conditions, PvuII can cleave DNA at sites that match only five of the six bases in the canonical PvuII cleavage site (Nasri & Thomas 1987, Robinson & Sligar 1995). However, such star activity has not been observed in vivo (Nastri et al. 1997, Simoncsits et al. 2001), and inspection of RET/PTC3 junctions found no evidence of breaks made by PvuII star activity. Furthermore, nonspecific cutting has not been shown for StuI. Therefore, the data do not support nonspecific cleavage by any known capability of PvuII.

In summary, in this study, we established a model of DSBs induced by various REs in human thyroid cells and report for the first time the formation of a carcinogenic chromosomal rearrangement after RE electroporation. The results obtained using this model suggest that complex DSB produced in one of the fusion partners may be sufficient for the formation of RET/PTC in thyroid cells. Moreover, this model can be used for further studies of radiation carcinogenesis in human thyroid cells, particularly in light of a similarity of RET/PTC types induced by RE and by radioiodine.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0314.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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