Role of H$_2$O$_2$ in RET/PTC1 Chromosomal Rearrangement Produced by Ionizing Radiation in Human Thyroid Cells

Rabii Ameziane-El-Hassani$^{1,2}$, Myriem Boufraqech$^{1,2,4}$, Odile Lagente-Chevalier$^{2,4}$, Urbain Weyemi$^{2,4}$, Monique Talbot$^{2,4}$, Didier Métivier$^{3,4}$, Françoise Courtin$^{2,4}$, Jean-Michel Bidart$^{2,4}$, Mohammed El Mizibri$^1$, Martin Schlimmer$^{2,4}$, and Corinne Dupuy$^{2,4}$

Abstract

During childhood, the thyroid gland is one of the most sensitive organs to the carcinogenetic effects of ionizing radiation that may lead to papillary thyroid carcinoma (PTC) associated with RET/PTC oncogene rearrangement. Exposure to ionizing radiation induces a transient "oxidative burst" through radiolysis of water, which can cause DNA damage and mediate part of the radiation effects. H$_2$O$_2$ is a potent DNA-damaging agent that induces DNA double-strand breaks, and consequently, chromosomal aberrations. Irradiation by 5 Gy X-ray increased extracellular H$_2$O$_2$. Therefore, we investigated the implication of H$_2$O$_2$ in the generation of RET/PTC1 rearrangement after X-ray exposure. We developed a highly specific and sensitive nested reverse transcription-PCR method. By using the human thyroid cell line HTori-3, previously found to produce RET/PTC1 after γ-irradiation, we showed that H$_2$O$_2$, generated during a 5 Gy X-ray irradiation, causes DNA double-strand breaks and contributes to RET/PTC1 formation. Pretreatment of cells with catalase, a scavenger of H$_2$O$_2$, significantly decreased RET/PTC1 rearrangement formation. Finally, RET/PTC chromosome rearrangement was detected in HTori-3.1 cells after exposure of cells to H$_2$O$_2$ (25 μmol/L), at a dose that did not affect the cell viability. This study shows for the first time that H$_2$O$_2$ is able to cause RET/PTC1 rearrangement in thyroid cells and consequently highlights that oxidative stress could be responsible for the occurrence of RET/PTC1 rearrangement found in thyroid lesions even in the absence of radiation exposure.

Cancer Res; 70(10); 4123-32. ©2010 AACR

Introduction

Chromosomal rearrangements involving the RET gene (REArranged during Transfection) are highly prevalent in papillary thyroid carcinomas (PTC) occurring after childhood exposure either to environmental radiation after the Chernobyl accident or to medical external radiation (1–4). In the normal thyroid gland, RET which encodes the tyrosine kinase receptor for growth factors belonging to the glial-derived neurotrophilic factor family, is expressed only in parafollicular thyroid cells. The recombination of the intracellular kinase encoding domain of the RET gene with a heterologous gene expressed ubiquitously generates RET/PTC chimeric oncogene proteins that are expressed in follicular thyroid cells. These chimeric proteins are constitutively active (1, 2). RET/PTC1, which is the most frequent rearrangement, is the result of the fusion of RET and CCDC6 genes from a paracentric inversion of the long arm of chromosome 10 with breakpoints in q11.2 and q21 [inv(10) (q11.2q21); ref. 5]. Despite the fact that RET and CCDC6 genes are 30 mb apart, their high spatial proximity in interphase nuclei in normal thyroid cells was thought to promote their recombination (6, 7).

External radiation during childhood increases the risk of thyroid tumors, either benign or malignant (1–4, 8). More than 90% of these cancers are papillary, presenting a RET/PTC rearrangement in 70% of cases. Several in vitro and in vivo studies have shown that exposure to ionizing radiation induces RET/PTC rearrangement. Thus, using a nested PCR assay, RET/PTC1 transcripts were detected in a human undifferentiated thyroid carcinoma cell line (8505C) after exposure to 50 Gy of X-rays (9). RET/PTC1 and RET/PTC3 were detected in human thyroid tissues transplanted in severe combined immunodeficiency mice and submitted to X-ray irradiation (10). More recently, RET/PTC rearrangements were induced in immortalized human thyroid cells (HTori-3) after exposure to γ-radiation in a dose-dependent manner (11). These results strongly support the direct role of radiation exposure in RET/PTC generation.

Irradiating radiation exposure induces a transient "oxidative burst" through water radiolysis (12) that generates H$_2$O$_2$, and in turn, H$_2$O$_2$ could cause DNA damage and mediate part of...
the radiation effects (12). H₂O₂ can induce DNA double-strand breaks (DSB) and consequently chromosomal aberrations (13–16).

In this study, we show for the first time that H₂O₂ produced during irradiation but also when it is added to the cell medium, causes RET/PTC1 chromosomal rearrangements in human thyroid cells. Consequently, oxidative stress could be responsible through the generation of H₂O₂ for the occurrence of RET/PTC1 rearrangements that may lead to thyroid tumors, even in the absence of radiation exposure.

Materials and Methods

Cell culture conditions
The human thyroid epithelial cell line (HTori-3), purchased from the European Tissue Culture collection, was grown in phenol red–free RPMI 1640 (Invitrogen, Inc.), supplemented with 1% (v/v) antibiotics/antimycotics (Invitrogen), 2 mmol/L of l-glutamine (Invitrogen), and 10% (v/v) FCS (PAA Laboratories). The human thyroid papillary carcinoma cell line (TPC-1) was cultured in the same medium. The human embryo lung fibroblast (MRC5) cell line, kindly given by Dr. Filippo Rosselli (Fre 2939, Centre National de la Recherche Scientifique, Villejuif, France), was cultured in phenol red–free DMEM (Invitrogen) supplemented with 1% (v/v) antibiotics/antimycotics, 2 mmol/L of l-glutamine, 1 mmol/L of sodium pyruvate (Invitrogen), and 10% FCS. Normal human thyroid tissue specimens were collected at Institut Gustave Roussy in accordance with local and national ethics laws. Informed consent was obtained from all patients. Primary human thyrocytes were cultured as previously described (17).

Expression of thyroid differentiation markers
Total RNA from cells was extracted using Trizol reagent (Invitrogen) according to the instructions of the manufacturer and reverse transcription was done as previously described (17). cDNA was then amplified by 40 temperature cycles (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute) in a GeneAmp 9600 apparatus and AmpliTaq Gold’s protocol (Applied Biosystems). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA was amplified as a control. Selected primers are listed in Table 1.

Transient cell transfection
HTori-3 cells reaching 50% to 60% confluence were transfected using JetPEI transfection reagent and the Polyplus Cyto vector (Evrogen JSC). After 48 hours, the cells were visualized by confocal microscopy.

Confocal fluorescence microscopy
pHyPer-transfected cells were plated on glass-bottomed dishes, exposed to 5 Gy of X-ray irradiation or to 25 μmol/L of H₂O₂, and immediately analyzed by confocal microscopy. The fluorescence (excitation/emission, 488/530 nm) was examined under an LSM 510 confocal microscope (Zeiss).

Cell treatment
X-ray irradiation. In most experiments, 5 × 10⁶ cells were plated in a 175 cm² flask and 16 hours later exposed to a single dose of X-irradiation from an X-ray Generator operating at 200 kV and 15 mA at a dose rate of 1 Gy/min. Just before irradiation at room temperature, the cell medium was replaced with fresh medium. Four hours after irradiation, cells were collected by trypsinization and split into 10 six-well plates at a density of ~2.2 × 10⁴ cells per well, grown for 15 days, and harvested.

Hydrogen peroxide. For RET/PTC1 detection, 5 × 10⁶ cells were plated in a 175 cm² flask and 16 hours later exposed to 25 μmol/L of H₂O₂ in serum-free medium for 1 hour at 37°C. Four hours later, cells were collected by trypsinization and split into 10 six-well plates at a density of ~2.2 × 10⁴ cells per well, grown for 15 days, and harvested.

Detection of RET/PTC1 rearrangements
The Total RNA from irradiated cells cultured in six-well plates was extracted using a NucleoSpin RNA II kit (Macherey-Nagel) according to the protocols of the manufacturer. The integrity and purity of each RNA sample was verified with the Agilent 2100 Bioanalyzer (Agilent Biotecnologies). The RET-specific reverse transcription was achieved by using a gene-specific primer (RT-RET-994) in the presence of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The first and the nested PCR were carried out with 35 cycles in the respective conditions (95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute) and (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) in a GeneAmp 9600 apparatus using a protocol from AmpliTaq (Applied Biosystems). For optimization of the first-round PCR conditions, three target reverse transcriptions from each sample were followed by three first PCRs. Briefly, 6 μg of RNA from each sample was divided into 2 μg aliquots, dispensed in 10 μL of reverse transcription buffer, and incubated for 120 minutes at 37°C according to the protocols of the manufacturer. Each reaction mixture was then diluted with 14 μL of the PCR buffer containing 0.5 units of Taq DNA polymerase (Applied Biosystems, Roche). The first-round reverse transcription-PCR (RT-PCR) products were pooled, diluted 1:5, and then 5 μL were used for one nested PCR. The PCR amplification products were analyzed with 1.5% agarose gel electrophoresis and ethidium bromide staining. The specificity of the amplification product with predicted size was confirmed by EcoRl digestion. The primers used are listed in Table 1.

Measurement of H₂O₂ generation
Production of H₂O₂ was determined by reaction with cell-impermeable 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent, Invitrogen) in the presence of excess peroxidase, producing fluorescent resorufin. Briefly, HTori-3 cells were plated into six-well plates at a density of 2.5 × 10⁵ per well in complete medium. Sixteen hours later, the cells were washed twice with warm Dulbecco’s PBS (D-PBS) without CaCl₂ and MgCl₂ (Invitrogen) and irradiated by 5 Gy of X-ray in D-PBS with CaCl₂ and MgCl₂ (Invitrogen). Cell-free
The HTori-3 cell line has been successfully used (11) to study the occurrence of RET/PTC rearrangement after in vitro radiation exposure. In spite of the presence of multiple numerical and statistical analyses, the results were significant only when the threshold was set at P < 0.05.

Table 1. Sequence of the selected primers and predicted size of PCR product

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sense</th>
<th>5′–3′ Sequence</th>
<th>PCR products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human TPO</td>
<td>+</td>
<td>CCGGTCTCATCTGTGACAAACAC</td>
<td>448 bp</td>
</tr>
<tr>
<td>Human Tg</td>
<td>–</td>
<td>CGGAGCTTAGGGTGGTTC</td>
<td>445 bp</td>
</tr>
<tr>
<td>Human TSH-receptor</td>
<td>–</td>
<td>GCCAAAGGGTGCTGGAAGTC</td>
<td>418 bp</td>
</tr>
<tr>
<td>Human G3PDH</td>
<td>+</td>
<td>CTGGCAGAAGCCAAGCTCCTC</td>
<td>452 bp</td>
</tr>
<tr>
<td>Human external RET/PTC1</td>
<td>+</td>
<td>CAAAGAGAACAGGTGCTGGAAG (CCDC6 exon 2)</td>
<td>283 bp</td>
</tr>
<tr>
<td>Human internal RET/PTC1</td>
<td>–</td>
<td>GCCAGGTCGAAGCTCCTC (RET exon 12)</td>
<td>212 bp</td>
</tr>
<tr>
<td>RT-RET-994</td>
<td>+</td>
<td>CACTTTGCCTGCTGTA</td>
<td>241 bp</td>
</tr>
</tbody>
</table>

NOTE: +/- means that the oligonucleotide is sense or antisense in relation to cDNA.

The peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein (DCFDA; Molecular Probes) was used to assess the generation of intracellular reactive oxygen species (ROS). This compound is converted by intracellular esterases to 2′,7′-dichlorodihydrofluorescein, which is then oxidized by intracellular ROS to the highly fluorescent 2′,7′-dichlorodihydrofluorescein, which was used to assess the generation of intracellular reactive oxygen species (ROS). This compound is converted by intracellular esterases to 2′,7′-dichlorodihydrofluorescein (DCF). HTori-3 cells were plated at a density of 105 cells per well into six-well plates in complete culture medium. Sixteen hours later, the cells were harvested by trypsinization, resuspended in 0.5 mL of serum-free medium and incubated with 10 μmol/L of CM-H2DCFDA for 1 hour at 37°C in serum-free medium in the dark before being treated with 25 μmol/L of H2O2. Relative fluorescence units (excitation/emission, 485/535 nm) were measured in a multiwell fluorescence plate reader (Wallac 1420 Victor2, Perkin-Elmer).

Cell survival test

Cell proliferation/viability of HTori-3 cells (96-well plates; 3 × 104 cells/well) was quantified by means of a colorimetric assay based on the reduction of the colorless tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, Roche Applied Science) to formazan, according to the instructions of the manufacturer.

SDS-PAGE and Western blotting procedures

Western blot analysis was performed with lysates prepared as previously described (17). Primary antibody against phosphorylated Ser139 of histone H2AX (γH2AX) was purchased from Abcam. The secondary antibodies used were, respectively, horseradish peroxidase–conjugated anti-mouse (Santa Cruz) and horseradish peroxidase–conjugated goat anti-rabbit (Southern Biotech). Specific binding was detected by the Enhancer Chemiluminescence system.

Statistical analysis

Data are means ± SEM. Student’s t test was used with P < 0.05 as the significant threshold.

Results

In vitro irradiation of the HTori-3 cells induced RET/PTC formation

The human thyroid epithelial cell line. The HTori-3 cell line has been successfully used (11) to study the occurrence of RET/PTC rearrangement after in vitro radiation exposure. In spite of the presence of multiple numerical and statistical analyses, the results were significant only when the threshold was set at P < 0.05.
structural chromosomal abnormalities, these cells present some advantages: they have three intact copies of chromosome 10 with undisrupted RET locus and a high proliferation rate, both increasing the probability of RET/PTC formation after radiation exposure (11, 18). As previously shown, the HTori-3 cell line expresses mRNA of thyroid differentiation markers such as thyroglobulin (Tg), thyroperoxidase (TPO), and TSH receptor, which are positively regulated by TSH (Fig. 1A). Primary cultures of human thyrocytes were used as a control. Unlike thyrocytes, HTori-3 cells did not produce extracellular H$_2$O$_2$ under stimulated and nonstimulated conditions (data not shown).

**Detection of RET/PTC1 rearrangement.** To detect RET/PTC1, we developed a highly specific and sensitive nested RT-PCR method by using first a gene-specific primer for reverse transcription and second two couples of primers designed, respectively, from CCDC6 exon 2 and RET exon 12 (Table 1). Dilution experiments were carried out mixing TPC-1 cells bearing the RET/PTC1 rearrangement with untreated HTori-3 cells in which RET/PTC1 rearrangement was undetectable (Table 2) to evaluate the sensitivity of the method. RNA was extracted from the mixture and subjected to nested RT-PCR. A 212 bp product could be detected from the TPC-1 cells when mixed with HTori-3 cells at a ratio of 1:10$^6$ (Fig. 1B). The specificity of RET/PTC1-positive amplified product was ascertained by EcoRI digestion of the PCR products that resulted in the predicted fragments (Fig. 1C). Then, RET/PTC1 rearrangement was sought in HTori-3 cells after X-ray exposure. Representative ethidium bromide-stained gels of RT-PCR products are shown in Fig. 1D. To exclude the possibility of degradation of extracted RNA, G3PDH was amplified from each sample using RT-PCR as a control. Because the highest number of RET/PTC1 was observed after 5 Gy of γ-radiation in cells grown for 9 days after irradiation (11), the cells were irradiated with a single dose of 5 Gy X-ray, 4 hours later, seeded in six-well plates and collected after 15 days. In these conditions, RET/PTC1 rearrangement was detected in ~20% of cell-containing wells.
addition of 100 units/mL of catalase, a scavenger of H_2O_2,
and only 50% remained at 10 minutes after irradiation. The concentration of H_2O_2 produced in the medium through transient oxidative burst through radiolysis of water (12). The extracellular medium through radiolysis of water suppressed radiation-induced H_2O_2 formation in the cell HTori-3 cell line is a valuable in vitro (mean, 20.53 ± 1.98; Table 2). Thus, we confirmed that the HTori-3 cell line is a valuable in vitro model for studying the mechanisms of RET/PTC1 generation. The RET/PTC1 signal intensity in the positive wells seemed comparable to the signal in TPC1 lines because the nested PCR conditions were not compatible with quantification. Indeed, the initial, outer primer PCR phase was not conducted in the exponential phase. No transcripts of RET/PTC1 rearrangement were detectable in the nonthyroid irradiated MRC5 cell line and were analyzed in the same experimental conditions of radiation exposure (Table 2), showing that RET/PTC1 rearrangement does not occur in fibroblasts.

**Ionizing radiation exposure produces H_2O_2 in the extracellular medium through radiolysis of water**

Ionizing radiation exposure is known to induce a transient oxidative burst through radiolysis of water (12). The concentration of H_2O_2 produced in the medium through radiolysis of water after irradiation with 5 Gy of X-ray was measured at different time intervals. In the absence of cells, the concentration estimated at 12 μmol/L remained unchanged 30 minutes after irradiation (Fig. 2A). On the other hand, in the presence of cells, H_2O_2 rapidly disappeared and only 50% remained at 10 minutes after irradiation. The addition of 100 units/mL of catalase, a scavenger of H_2O_2, suppressed radiation-induced H_2O_2 formation in the cell medium.

**Pretreatment of thyroid cells by catalase decreases the occurrence of DNA DSBs and of RET/PTC1 rearrangement after exposure to ionizing radiation**

Ionizing radiation exposure is known to induce several kinds of DNA damage. DNA DSBs is the main cytotoxic lesion induced by ionizing radiation. The phosphorylated form of histone H2AX (γH2AX) at Ser139 is generally used as a marker of DNA DSBs (19, 20). As shown in Fig. 2B, HTori-3 cells displayed phosphorylation of histone H2AX at 5 minutes after exposure to 5 Gy of X-ray irradiation that increased up to 1 hour. A genetically encoded highly specific fluorophore probe has been recently developed for detecting H_2O_2 inside living cells (21). This indicator, named HyPer, has been shown to have submicromolar affinity to H_2O_2 and to be insensitive to other oxidants. Using the mammalian expression vectors encoding, respectively, cytosolic- and nuclear-targeted HyPer, we visualized changes in the fluorescence of HyPer in both the cytosol and the nucleus upon irradiation of the cells, suggesting that an increase of the H_2O_2 levels occurred following radiation exposure and might be responsible for some of the effects of radiation (Fig. 2C). To confirm the potential role of H_2O_2 protection of DNA from oxidative damage was evaluated by pretreating HTori-3 cells with catalase at different times before irradiation. Whereas the presence of catalase in the culture medium during irradiation partially protected the cells from DNA damage, preincubation of the cells for 4 hours with catalase significantly decreased the level of phosphorylated H2AX as shown by Western blotting (Fig. 2D). This result confirmed by pulse-field gel electrophoresis, a sensitive assay for DNA DSBs, showed that preincubation of HTori-3 cells for 4 hours with catalase protected DNA from the occurrence of DSBs and significantly decreased the occurrence of RET/PTC1 rearrangements (Table 2).

**Extracellular H_2O_2 is able to cause RET/PTC1 rearrangements in thyroid cells**

The effect of H_2O_2 concentrations from 1 to 25 μmol/L on the intracellular ROS level was determined by flow cytometry analysis of DCFH-DA oxidation. One hour after treatment with H_2O_2, we measured a concentration-dependent increase of fluorescence, reflecting an increased level of intracellular ROS (Fig. 3A). Preincubation of HTori-3 cells with catalase for at least 4 hours was necessary to produce a significant decrease in intracellular level of ROS induced after 25 μmol/L of H_2O_2 treatment (Fig. 3B). Using confocal

### Table 2. RET/PTC1 transcript formation in irradiated and surviving HTori-3 and MRC5 cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Wells tested</th>
<th>Wells RET/PTC1 positive</th>
<th>RET/PTC1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTori-3</td>
<td>Experience 1 Control</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Experience 1 5 Gy</td>
<td>51</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Experience 2 5 Gy</td>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Experience 3 5 Gy</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>MRC5</td>
<td>Experience 1 Control</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Experience 1 5 Gy</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Experience 1 5 Gy + catalase</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Experience 2 5 Gy + catalase</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Experience 3 5 Gy + catalase</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>HTori-3</td>
<td>Experience 1 H_2O_2 25 μmol/L</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Experience 2 H_2O_2 25 μmol/L</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Experience 3 H_2O_2 25 μmol/L</td>
<td>60</td>
<td>8</td>
</tr>
</tbody>
</table>

**NOTE:** Effect of the catalase pretreatment on the RET/PTC1 incidence in irradiated surviving HTori-3 cells. RET/PTC1 (mean, 20.53 ± 1.98; Table 2).
microscopy, after the addition of 25 μmol/L of H2O2 in the cell culture medium, we followed the changes in fluorescence of HyPer expressed in the nucleus. After 2 minutes, we observed an increase in fluorescence indicating an H2O2-induced signal in the nucleus (Fig. 3C). Preincubation of cells for 4 hours with catalase significantly decreased the nuclear fluorescence.

As shown in Fig. 4A, H2O2 concentrations ≤25 μmol/L did not induce any significant cell death after 24 hours. Time course analysis of γH2AX in response to 12 and 25 μmol/L of H2O2 in HTori-3 cells showed that H2AX was highly phosphorylated after 1 hour (Fig. 4B). The phosphorylation of H2AX at Ser139, which was correlated to the H2O2 concentration, confirmed that 12 μmol/L of H2O2, corresponding to the concentration measured after radiation exposure, was able to cause DNA damages. As observed for 5 Gy of X-ray irradiation, preincubation of cells with catalase for 4 hours protected DNA from DSBs produced after exposure to 12 μmol/L of H2O2 for 1 hour (Fig. 4C).

In the light of these findings, we treated the cells with 25 μmol/L of H2O2 for 1 hour, which was found to cause the highest level of DNA DSBs without affecting the cell viability and evaluated the occurrence of RET/PTC1 rearrangements in HTori-3 cells 2 weeks later by nested RT-PCR. As shown in Table 2 and Fig. 4D, 25 μmol/L of H2O2 was able to produce RET/PTC1 chromosomal rearrangements.
**Discussion**

To our knowledge, this is the first report showing that H$_2$O$_2$ promotes RET/PTC1 rearrangements in thyroid cells, and consequently, these data confirm that H$_2$O$_2$ might play an important role in the initiation of PTCs. The direct link between ionizing radiation, RET/PTC rearrangements and PTC occurrence has been supported by the observation that RET/PTC rearrangements are frequently found in PTC samples from patients who had been exposed during childhood to external radiation or who lived in highly contaminated regions after the Chernobyl accident (22). In addition, several studies have shown that in vitro irradiation of thyroid cells is able to cause RET/PTC1 rearrangements (9, 10). In a recent study, RET/PTC rearrangements were induced in differentiated human thyroid cells by a range of γ-radiation doses (0.1–10 Gy), that were equivalent to those received by patients who developed a thyroid cancer (11). By using the same experimental model, we also identified RET/PTC1 rearrangements in thyroid cells grown for 15 days after in vitro exposure to a single X-radiation dose of 5 Gy.

Chronic oxidative stress may disrupt the genomic integrity of cells and might induce chromosomal instability (14–16, 23). For many years, H$_2$O$_2$, considered a DNA genotoxic agent, was suspected to play a mutagenic role in the thyroid gland and to be responsible for the thyroid nodules that were frequently found (24). Several observations strengthen this hypothesis: in mice, the spontaneous mutation frequency is higher in the

![Figure 3. Effect of extracellular H$_2$O$_2$ exposure on intracellular ROS levels.](image)

A, effect of H$_2$O$_2$ on ROS levels measured as DCF fluorescence by flow cytometry in HTori-3 cells incubated after 1 hour with different concentrations of H$_2$O$_2$. B, effect of catalase on ROS levels in HTori-3 cells treated with 25 μmol/L of H$_2$O$_2$. Catalase (100 units/mL) was preincubated for 2 and 4 hours before irradiation. ROS levels were measured as DCF fluorescence in a multiwell fluorescence plate reader as described in Materials and Methods. C, nuclear fluorescence intensities of HyPer in cells exposed to 25 μmol/L of H$_2$O$_2$ as measured by confocal microscopy.
thyroid gland than in any other organ (25), somatic mutations of the TSH receptor are frequent in human thyroid tissue (24), and thyroid glands of mice transgenic for the mutant α1B-adrenergic receptor (Tg-α1BAR mice) that have increased H2O2 generation develop malignant nodules with high frequency (26). H2O2 causes DNA damage, such as guanine oxidation, single-strand breaks and DSBs leading to mutagenesis and genomic instability. DNA DSBs (27) that can lead to chromosomal aberrations (28) result from both a direct effect of irradiation on DNA and from the generation of ROS by radiolysis of water (12). Although DSBs induced by H2O2 are considered rare events, it has been shown that H2O2 induces DSBs with a ratio of DSBs to single-strand breaks similar to that caused by X-rays (13, 29). This induction of DSBs by H2O2 was also observed in rat thyroid cell line (PCC13), in human thyroid primary culture and pig thyroid slices (13). However, in these experiments, the H2O2 concentrations used were at least five times higher than those used herein to generate DSBs in the human thyroid cell line HTori-3. This difference is probably due to the models used, which have different cell membrane permeability and antioxidant capacities. HTori-3 cells seem to be particularly sensitive to H2O2, because 50 μmol/L of H2O2 treatment affected the viability of these cells. Our results showed that extracellular H2O2 produced through water radiolysis significantly contributes to the appearance of DSBs, and importantly, to RET/PTC1 generation. The extracellular concentration of H2O2 measured after 5 Gy of irradiation was estimated at 12 μmol/L and was shown to cause DNA damage. This result was in disagreement with the recent report of very low H2O2 concentrations after irradiation of the cell medium by similar γ-ray doses and of the absence of a potentiating effect of L-buthionine-sulphoximine on DNA damage, a drug that decreases intracellular glutathione, and consequently, induces a protective effect for H2O2 against degradation (13). This discrepancy could be due to our experimental conditions, because serum was withdrawn from the cell medium before irradiation to avoid H2O2 produced during radiation exposure to be degraded by the catalase activity of the serum (30).
It was previously observed that extracellular H$_2$O$_2$ rapidly disappeared (13, 31). We also found that 90% of the extracellular H$_2$O$_2$ measured in the cell medium after irradiation had disappeared after 20 minutes. As measured by DCF fluorescence in the cells, the intracellular production of ROS was correlated with the concentrations of exogenous H$_2$O$_2$ added in the medium. Importantly, these H$_2$O$_2$ concentrations ranged from 1 to 25 μmol/l and were of the same order as those measured in the medium after radiation exposure, and were shown to produce DNA damage. Using HyPer, a specific probe for H$_2$O$_2$, we detected a temporal increase in H$_2$O$_2$ in the nucleus of cells treated with exogenous H$_2$O$_2$. These results confirm that extracellular H$_2$O$_2$ diffuses into the cells, reach the nucleus, and consequently, might cause DNA damage. Catalase was able to protect cells from the toxic effects of H$_2$O$_2$, demonstrating for the first time that H$_2$O$_2$ produced during irradiation contributes to the generation of RET/PTC1 in thyroid cells. In the present study, we observed that pretreatment of the cells with catalase for at least 4 hours produced a significant inhibition of DNA DSBs induced by irradiation as shown by phosphorylation of histone H2AX. The protective effect of pretreatment with catalase was associated with a significant decrease in intracellular and intranuclear levels of H$_2$O$_2$. As the oxidative-sensitive fluorescence dye (DCF) is not specific for H$_2$O$_2$ and could also be oxidized by other radicals produced in these conditions, the decrease observed was partial (32, 33). Thus, as observed in other studies (34), exogenous catalase could enter the cells by endocytosis, scavenge intracellular H$_2$O$_2$, and protect the cells from oxidative damage. However, it could also interact with the cell membranes in a time-dependent manner and scavenge extracellular H$_2$O$_2$ before its diffusion into the cells. These results corroborate previous results showing a protective effect of antioxidant treatments from ionizing radiation-induced malignant transformation and tumorigenicity of HTori-3 cells (35, 36).

The thyroid gland, in normal conditions, produces large amounts of H$_2$O$_2$ (37), that was estimated in stimulated rat thyroid cell lines and in rat thyroid cells in primary cultures to be ∼10 nmol/100 mg/h (38). The thyrocytes have two H$_2$O$_2$-generating systems located at the apical membrane: DUOX1 and DUOX2, which are NADPH oxidases (39, 40). Besides being important for hormone synthesis, DUOX could be a major source of free radicals and ROS that could cause substantial DNA damage. Recently, we showed that NOX4, another NADPH oxidase, is expressed in normal and cancer thyroid tissues and might be an H$_2$O$_2$ generator located inside the thyroid cell (17).

In this study, we show that H$_2$O$_2$ is able to cause RET/PTC1 chromosomal rearrangement, the most prevalent mutation detected in PTC. However, we cannot exclude that H$_2$O$_2$ could also cause other specific mutations found in the thyroid cancers as BRAF, Ras, and PAX8/PPARY.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Grant Support**

Electricité de France, Association pour la Recherche sur le Cancer, Ligue Contre le Cancer (comité du Val-de Marne), and Agence Nationale de la Recherche.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/01/2009; revised 03/03/2010; accepted 03/09/2010; published OnlineFirst 04/27/2010.

**References**

Role of H$_2$O$_2$ in $RET/PTC1$ Chromosomal Rearrangement Produced by Ionizing Radiation in Human Thyroid Cells


*Cancer Res* 2010;70:4123-4132. Published OnlineFirst April 27, 2010.

Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-4336

Cited articles This article cites 40 articles, 14 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/10/4123.full#ref-list-1

Citing articles This article has been cited by 8 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/70/10/4123.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.