Yttrium-90 Chimeric L6 Therapy of Human Breast Cancer in Nude Mice and Apoptosis-related Messenger RNA Expression

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Abstract

Radioimmunotherapy (RIT) in breast cancer patients using I-131-chimeric L6 (ChL6) and in human breast cancer xenografts in nude mice using Y-90-1,4,7,10-tetraazaacyclodecan N,N',N",N"-tetraacetic acid-peptide ChL6 (Y-90-ChL6) has shown promise. Tumor cell response to low-dose rate (5–25 rad/h) irradiation from Y-90-ChL6 RIT, therefore, was correlated with levels of tumor cell mRNA for selected genes linked to programmed cell death (apoptosis). Three groups of 10–16 mice with 1–2 HBT 3477 xenograft tumors were treated with 100, 150, or 250 μCi Y-90-ChL6. Three tumors were taken before and two tumors each were taken 3, 6, and 24 h after injection of 150 μCi Y-90-ChL6. Tumor expression of mRNA was amplified by PCR for p53, PICI, c-myc, and transforming growth factor-β1; quantitated; and standardized to N-ras.

Tumors received radiation doses of 2000, 3000, and 5000 rads, respectively, for the groups of mice that received 100, 150, and 250 μCi Y-90-ChL6, and tumor regression occurred in each group, with mean tumor volumes decreased by 10, 50, and 95% at nadir after Y-90-ChL6 injection. At the highest dose level, 30% of mice had complete remissions, and no treatment deaths occurred, although tumors subsequently recurred.

Continuous up-regulation of transforming growth factor-β1 and c-myc mRNA expression was observed from 3 to 24 h after treatment. Expression of p53 and PICI increased at 3 h and subsequently decreased to the untreated control levels. These observations are consistent with previous observations of early responses of p53 and PICI to cellular DNA damage and subsequent G1 cell cycle arrest or apoptosis.

Apoptosis-associated gene expression patterns observed in this tumor model provide evidence that changes are initiated in the first 24 h of RIT associated with radiation doses of 100–700 rads. These preliminary data suggest that insight into the molecular basis of RIT-induced tumor regression may be gained by further studies using different radiation doses.

Introduction

RIT is based on the ability to deliver systemic radiotherapy on molecules that concentrate preferentially in tumor tissue, resulting in a high therapeutic ratio between radiation dose: tumor relative to the normal tissue dose (1, 2). Tumor responses have been reported from multiple clinical trials using this type of therapy in patients with advanced lymphoma, leukemia, and breast cancer (3–7). Although tumor regressions are probably the result of the radiation dose alone, the extent of the response often exceeds that expected for the estimated radiation dose and dose rate (2). Although a growing body of information suggests that apoptosis (programmed cell death) may be the major mechanism of tumor cell death from low-dose rate radiation, this has not been proven or generally accepted (8–10). However, apoptosis has been recognized as a response of lymphoma and breast and prostatic cancer to several important cancer therapies, such as hormone withdrawal and DNA-damaging drugs (11–13). Recently, evidence has been found that various intracellular molecular pathways are related to apoptosis and converge to induce apoptosis in tumor cells (Fig. 1; Refs. 14 and 15).

To study the response of human cancer cells to RIT, we have chosen to initiate studies in breast cancer and to compare the molecular genetic response to that of previously described apoptotic pathways (11, 13, 14, 16). The selection of the specific mRNA for study was based on recent molecular studies of oncogenes and tumor suppressor genes, which have identified p53, PICI, c-myc, and TGF-β, as important components of altered apoptotic pathways in tumor cells. Apoptosis triggered by the p53 tumor suppressor gene, one of the most frequently mutated genes in human cancers, has been relatively well characterized (13, 17, 18). The normal wtp53 gene is essential for apoptotic cell death in various types of cells (the p53-dependent pathway) and acts by producing a DNA-binding protein, p53. This protein has both activating and repressing capabilities mediated through its transcriptional regulation of several target genes (19). One of the genes for which transcription is induced by wtp53 is PICI (CDKN1, WAF1/CIP1), an inhibitor of cyclin-dependent kinases (20). On exposure of cells to DNA-damaging agents, such as radiation or chemotherapy, PICI is induced by wtp53 but not by mutant type p53 expression resulting in a G1 cell cycle arrest (21, 22). On the other hand, little is known concerning the increased expression of mRNA from the TGF-β gene frequently found in cells undergoing apoptosis (23). Although its biological role in the apoptotic pathway is not well understood, it is possible that conflicting signals induced by increased TGF-β and c-myc might be a driving force for apoptosis when converging with signals from other molecular triggers (Fig. 1).

Knowledge of the timing and types of apoptotic pathways that are activated in vivo as a response of tumor cell-targeted low-dose-rate irradiation should provide insights into the molecular basis of this therapy that may lead to better methods of therapy. The MoAb ChL6 has shown promise for RIT in breast cancer patients as I-131-ChL6 (7). Recently, dosimetry from pharmacokinetic studies in nude mice suggested an enhanced therapeutic potential of the new radiopharmaceutical, Y-90-ChL6 (24). In this article we report Y-90-ChL6 therapy in nude mice bearing human breast cancer xenografts. Tumor growth in untreated animals is compared with that of treated animals, and timing of tumor regression after RIT is correlated with changes in gene expression.

Materials and Methods

Preparation of Therapeutic Radiopharmaceutical. ChL6 is an antitumor monoclonal antibody that reacts with breast, lung, colon, and ovarian cancers (25). The constant domains C-G2a and C-K of the mouse MoAb antibody have been replaced with human IgG1-equivalent domains (26). ChL6 was supplied at 5 mg/ml in normal saline by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA).

The bifunctional chelating agent, DOTA-peptide-NCS, was prepared according to the method previously described and labeled with Y-90 using a 1000:1 molar ratio of DOTA-peptide-NCS-Y-90 (24, 27, 28). The DOTA-peptide-NCS and Y-90 were incubated at 37°C for 60 min, and 50 mm diethylamineminaacetic acid in 0.1 M ammonium acetate, pH 6, was then added. Y-90-DOTA-peptide-NCS was purified from excess chelate and other
that were harvested, and mean tumor size obtained by caliper measurements determined for complete Y-90 decay. Actual tumor weight was used for tumors the sum of the radiation doses from tumor to tumor and from whole body to was used for each therapy group. The tumor radiation dose was calculated as tumor harvest, whereas that for the nonharvested tumors in all three groups was sampled for mRNA was determined from the time of injection to the time of studies to determine the cumulative activity. Cumulative activity for tumors were sampled for mRNA and for the remaining tumors that were used (untreated) group, and two tumors each were taken from mice sacrificed at 3, 6, and 24 h after receiving 150 @Ci Y-90-ChL6. At the time of sacrifice, expression levels of chosen genes. Three tumors were taken from the control (untreated) group, and two tumors each were taken from mice sacrificed at 3, 6, and 24 h after receiving 150 @Ci Y-90-ChL6. At the time of sacrifice, tumors removed for mRNA study were weighed, minced, and quick frozen. Cell Lines. HBT 3477 is a breast adenocarcinoma cell line obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. Estrogen and progesterone receptors were negative by immunopathology, and more than 70% of HBT 3477 cells stained for L in a Mwes type 1 pattern (29). The prostate adenocarcinoma cell line DU145 used as a gel standardization control was obtained from the American Type Culture Collection. Mouse Studies. Female athymic nu/nu mice on a BALB/c background, each weighing 17–20 g, received bilateral s.c. injections in their lower abdomens consisting of 5 × 10⁶ HBT 3477 cells. Tumors were measured in three dimensions with a caliper three times each week. Mice were preassigned to control or therapy groups. When tumors were 50–300 mm³, groups of mice were given 100, 150, or 250 @Ci Y-90-ChL6 by tail vein. Unlabeled ChL6 was added in amounts so that each mouse received a total of 315 @g ChL6. The 150-@Ci group of 16 mice had 26 tumors, and the 10 mice in the 100- and 250-@Ci groups had 16 and 20 tumors, respectively. A group of 13 mice with 23 tumors served as controls. Survival was monitored daily, and tumor size was monitored three times each week for 60 days after injection. Mice were sacrificed before and after therapy to obtain tumors for evaluation of mRNA expression levels of chosen genes. Three tumors were taken from the control (untreated) group, and two tumors each were taken from mice sacrificed at 3, 6, and 24 h after receiving 150 @Ci Y-90-ChL6. At the time of sacrifice, tumors removed for mRNA study were weighed, minced, and quick frozen. Radiation Dosimetry. To estimate the radiation doses for the tumors that were sampled for mRNA studies and for the remaining tumors that were used to evaluate tumor response to Y-90-ChL6 therapy, pharmacokinetic data for tumor and whole body observed in earlier studies were used (24). A monoexponential analysis of the pharmacokinetic data for the tumor and whole body was applied to the Y-90-ChL6 dose actually injected to the mice in these studies to determine the cumulative activity. Cumulative activity for tumors sampled for mRNA was determined from the time of injection to the time of tumor harvest, whereas that for the nonharvested tumors in all three groups was determined for complete Y-90 decay. Actual tumor weight was used for tumors that were harvested, and mean tumor size obtained by caliper measurements was used for each therapy group. The tumor radiation dose was calculated as the sum of the radiation doses from tumor to tumor and from whole body to tumor. The mean energy emitted per nuclear transition for Y-90 (S factor) was obtained from the medical internal radiation dose and corrected per Siegel and Stabin (30) for tumor to tumor and Coffey and Watson (31) for whole body to tumor.

RNA Extraction and cDNA Synthesis. Total cellular RNA was extracted from xenograft tumor specimens using the TRIzol RNA extraction kit (GIBCO BRL) according to the supplier’s instruction. One @g of extracted RNA was converted to cDNA by reverse transcription using random hexamer primers as previously described (32).

Quantitative RNA/PCR Analysis. The strategy for quantitative RNA/PCR used here is a further extension of our previous reports (32). Oligonucleotides used as amplification primers and probes are provided in Table 1. The sequences of oligonucleotides listed for p53, PIC1, and N-ras were described previously (17). To determine the optimal PCR cycling conditions for quantitation of each gene expression, RNA/PCR was initially performed under several different conditions including increasing cycle numbers. cDNA (amount generated from 25 ng of total RNA/100 µl PCR) for 34 cycles at 95°C (0.5 min), 58°C (0.5 min), and 72°C (0.5 min) was observed to be optimal amplification for all genes examined by the primers in this study except TGF-β1. For TGF-β1, 38 cycles and 1, 0.75, and 1.5 min proved optimal using 95, 58, and 72°C, respectively. Thus, cDNA prepared from tumor specimens was subjected to PCR amplification with each gene-specific primer set under the chosen cycling conditions. Triplicate PCRs were performed for each gene on every HBT 3477 tumor specimen and on the control cell line DU145. Ten to 15 µl RNA/PCR products were resolved by electrophoresis on 2% agarose gels with the corresponding triplicate RNA/PCR control gene product from DU145 and stained with ethidium bromide (0.5 µg/ml in 1x Tris-borate-EDTA). Quantitation of band intensities was performed by laser densitometry of the Polaroid negative of the original ethidium bromide-stained gels. Absolute area integrations of the curves representing each specimen were then compared after adjustment for the housekeeping gene (N-ras) expression. Integration and analysis were performed using the GelScan software program (LKB Pharmacia). Expression level ratios of the different target genes to N-ras in DU145 were set arbitrarily to 1.00 in each gel set of RNA/PCR, and the expression levels in tumor specimens were normalized using a correction factor (N-ras/target gene) determined from the standard DU145 from that gel. Thus, for each tumor specimen, expression levels were determined as follows: correction factor × mean of gene A/mean of N-ras. The maximum acceptable variance from the mean of the normalized triplicate RNA/PCR values from each tumor (for each gene) was set at 20%. An acceptable SD from the mean of the normalized values from the two tumors (three PCR reactions for each tumor) at each time point was set at 30%. This was based on our previous investigation of RNA/PCR variations (17).

To characterize the mutational status of the p53 gene in the HBT 3477 cell line, we performed nonisotopic RNA/PCR-SSCP analysis of p53 mRNA within exons 4–8 as previously described (17).

Results

Tumoricidal Effect. All tumors regressed in the mice that received Y-90-ChL6 (Fig. 2). Tumor growth curves of the treated mice began to deviate from those of the control mice beginning 4–7 days after treatment. Tumor volumes continued to decrease until the mean tumor volume of each treated group reached a nadir at 14, 16, and 38 days after treatment. The therapy dose (µCi) correlated with the amount

Table 1 Oligonucleotides used for RNA/PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs*</th>
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<tr>
<td>N-ras</td>
<td>CGGGGTTCCTCCTAATTTTTC</td>
</tr>
<tr>
<td>p53</td>
<td>GTATTGTTGTTCTCATGGCCACT</td>
</tr>
<tr>
<td>PIC1</td>
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<tr>
<td>TGF-β1</td>
<td>TTGGGCGAGTTGCTTCCTAGCGTGTC</td>
</tr>
<tr>
<td>c-myc</td>
<td>AGCTGGGGCCAGATTCCGAG</td>
</tr>
<tr>
<td></td>
<td>AGGCCTCTCTGAGGCGGGGCTTTTG</td>
</tr>
<tr>
<td></td>
<td>CACTTGAGGAGGGCAGATCATG</td>
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<tr>
<td></td>
<td>TTTTCGTCTTTCTCAGGGGC</td>
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<td></td>
<td>TTGGTTCTTCTCAGGCG</td>
</tr>
<tr>
<td></td>
<td>CTACTGTTGGAGGACATGGT</td>
</tr>
</tbody>
</table>

* All sequences are listed 5' to 3'.
Fig. 2. Tumor growth of HBT 3477 human breast cancer xenografts in four groups of nude mice: untreated (●) or treated with 100 (○), 150 (□), or 250 (△) μCi Y-90-ChL6. Each curve represents the group mean of the tumor volume from 10–16 mice bearing a total of 16–26 tumors. Tumors were taken for PCR studies from the 150-μCi therapy group at 3, 6, and 24 h after injection.

and duration of response (Fig. 2). No mice died due to the therapy at these dose levels. Although 3 of 10 mice at the 250 μCi dose level had complete remissions of their tumors, tumors recurred in each of these mice within 4 weeks of complete remission; thus, no mice were cured.

Radiation Dosimetry. Tumors received an average radiation dose of 2000, 3000, or 5000 rads, respectively, at the three Y-90 dose levels. The estimated radiation doses to the tumors harvested for mRNA expression at 3, 6, and 24 h after therapy were approximately 100, 200, and 700 rads, respectively.

Analysis of Apoptosis-associated Gene Expressions. Two HBT 3477 breast carcinoma xenograft tumors from each time point (3, 6, and 24 h after 150 μCi Y-90-ChL6) were characterized by quantitative RNA/PCR analysis and compared with the analysis of the three untreated specimens. A representative example of c-myc gene expression analysis by triplicate RNA/PCR and gel electrophoresis is shown in Fig. 3. Consistent levels of mRNA expression of each gene were observed in individual tissue specimens resected at the same time points. The variation in gene expression levels from replicates of the samples from a single time point for all genes reported was less than 15%, with the exception of the PICI levels at the zero time point, which approached 27% variation. For one of the two tumors taken at the 6-h time point, repeated triplicate PCR runs for c-myc and TGF-β1 had a variation in normalized gene expression exceeding the 20% limit defined in “Materials and Methods.” Although c-myc and TGF-β1 values for the other tumor at this time point were in keeping with results of other posttreatment tumors, this was not considered sufficient data to report a 6-h result for these two genes.

In treated tumors, an early increase in gene expression of p53 was identified, and its downstream gene, PICI, was even more distinctly increased. At 3 h after treatment, increases of approximately 1.4- and 1.6-fold in p53 and PICI, respectively, were observed (Fig. 4, A and B). Increased expression of p53 and PICI seemed to be downregulated to control levels by 6 h.

A continuous but modest rise of TGF-β1 was observed up to 24 h after treatment (Fig. 4D). In contrast, c-myc expression showed an earlier response, increasing in the tumors 3 h after treatment when the
radiation dose was only 100 rads. A further increase was observed at 24 h when the tumors had reached 700 rads (Fig. 4C).

On the RNA/PCR-SSCP analysis of p53 in untreated HBT 3477 cells, migration shifts of single-stranded DNA molecules were not found within the exonic areas in which the majority of p53 mutations are observed in human cancers (17, 18, 33). Thus, there is no evidence of sequence abnormalities in the p53 gene, although deletion of one allele could not be excluded.

Discussion

Clinical trials have shown RIT to be an effective treatment for some types of lymphomas and leukemias (3–6). Furthermore, modest responses to RIT in patients with advanced breast cancer have been documented (7, 34, 35). Although cellular damage induced in the tumor by irradiation is likely responsible for the major portion of tumor cell death, the extent of responses associated with relatively low radiation doses and dose rates emphasizes the need to understand the mechanisms involved. Recently, apoptosis has been recognized as a critical process not only in turnover of embryonal and replicating normal adult tissue, but also in the response of cancer cells to a variety of factors, including radiation and chemotherapy. Apoptosis can be induced by radiation, DNA-damaging drugs, binding to specialized receptors, and growth factor withdrawal (11–13, 16, 36, 37). There is evidence that portions of the molecular pathways involved in the induction of apoptosis may be different for different stimuli, although common genes also have been identified, such as p53 and c-myc (Fig. 1). Understanding the molecular response of tumor cells to RIT could lead to selection of combined therapies that enhance tumor responses.

To study the response of human breast cancer cells to RIT, the studies reported herein were performed in a nude mouse xenograft tumor model. Tumor regression correlated with the Y-90 dose level in groups of mice treated with 100, 150, or 250 μCi Y-90-ChL6. In the 150-μCi Y-90-ChL6 group, expression profiles of selected apoptosis-associated genes were analyzed for tumors harvested in the first 24 h after therapy. These tumors showed evidence of increased mRNA expression of several apoptosis-associated genes in the time preceding changes in tumor growth (Fig. 2). Levels of TGF-β1 and c-myc gene expression increased up to 24 h after treatment, and a transient increase in p53 and PIC1 expression occurred at 3 h after treatment and returned to control levels by 24 h.

In many studies of DNA-damaging agents (radiation and chemotherapy), an increase in p53 gene expression and the p53 protein has been seen shortly after treatment (21, 22). Studies also have shown that treatment of cells with these DNA-damaging agents resulted in increased expression of PIC1 in wtp53-containing cells but not in mutant type p53-containing cells. Increased expression of PIC1 in this study as well as the results of the RNA/PCR-SSCP analysis of these HBT 3477 cells indicate a wt status of the p53 gene in this tumor, although we cannot exclude the possibility of non-p53-dependent PIC1 expression (38). El-Deiry et al. (21, 22) also have found induction of PIC1 expression in cells undergoing p53-associated apoptosis but not in cells induced to undergo apoptosis through p53-independent mechanisms. Increased p53 protein and PIC1 mRNA were detected 2 h after irradiation in cells that underwent apoptosis subsequently 15 h later, although higher dose rates were used than those delivered by RIT in this study (22). In another study, Kastan et al. (39) observed the rapid increase of p53 protein in human ML-1 cells within 30 min after in vitro irradiation, and the levels returned to normal 48–72 h later. Results from both of these studies seem consistent with the patterns of p53 mRNA we observed in this in vivo study.

The molecular pathways by which the c-myc gene can function as an oncogene driving proliferation, and also as a potent inducer of apoptosis, are unclear. In the in vivo study presented in this article, we observed a rapid and continuous increase of c-myc expression in xenograft tissue specimens after treatment but prior to tumor shrinkage. This is consistent with several previous reports using well-defined in vitro models of apoptosis. By using complementary leucine zipper mutants, Amati et al. (40) found that c-myc can control two alternative cell fates, cell cycle proliferation and apoptosis, through dimerization with a single partner, the Max protein. There also is evidence that apoptosis triggered by withdrawal of either serum growth factors or specific hormones is correlated with increased
Acknowledgments

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References


13. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis is triggered converging pathways, and the appropriate timing for such growth cell populations may lead to useful combinations of agents therapy are being evaluated, because the TGF-β1 and c-myc may con


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