Noninvasive Imaging of the Transcriptional Activities of Human Telomerase Promoter Fragments in Mice

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ABSTRACT

We have assessed the feasibility of positron emission tomography (PET) and ex vivo γ-counting to measure the pattern of expression of telomerase promoter fragments in vivo. Promoter fragments from either the RNA [human telomerase RNA (hTR)] or the catalytic components [human telomerase reverse transcriptase (hTERT)] of the telomerase genes were used to drive the expression of the sodium iodide symporter PET reporter gene in recombinant adenoviruses. Both promoter fragments provided cancer-selective expression that could be visualized and quantitated by PET. The transcriptional activity of the hTERT promoter was found to be consistently stronger than that of the hTERT promoter. Both promoters appear therefore to be good candidates for safe use in gene therapy, and PET imaging could be used to assess the selectivity of promoters in vivo. Given that this methodology is directly scalable to humans, imaging gene expression using the sodium iodide symporter PET reporter gene could be applied to measure telomerase promoter activity in humans.

INTRODUCTION

Telomerase is essentially inactive in most differentiated or committed somatic cells whereas cancer cells need to maintain telomerase activity to achieve continued proliferation (1). This necessity is illustrated by the fact that telomerase is reported to be the most extensive tumor molecular marker (1–3), with high levels of telomerase activity in >85% of solid tumors. These observations have highlighted the importance of telomerase as a target and open therapeutic windows for a selective action on cancer cells (reviewed in Ref. 4). Direct inhibition of telomerase activity in cancer cells has been proposed and remains an important strategy. The mechanism of action of these inhibitors predicts that their cytotoxicity is likely to be delayed until telomere erosion reaches a threshold that would trigger senescence and/or apoptosis. Another proposed strategy takes advantage of the aberrant telomerase genes expression to design gene therapy formulations in which promoter fragments from either the RNA component [human telomerase RNA (hTR)] or the catalytic protein component [human telomerase reverse transcriptase (hTERT)] of the telomerase would be used to direct the expression of a therapeutic transgene. In this approach, telomerase-directed cell death is rapid. The efficacy of the latest approach has been demonstrated in vitro and in vivo using various gene therapy formulations in preclinical models (5–9). To translate these successful preclinical studies into early phase clinical trials, there is considerable value in the evaluation of the pattern of vector expression in terms of location and magnitude of expression.

These issues can be addressed through noninvasive imaging systems developed to detect transgene expression in vivo using positron emission tomography (PET), γ-camera, or bioluminescence imaging (see Ref. 10 for review). We and others have demonstrated that the activity of promoters can be measured in vivo by PET using marker genes such as the herpes simplex virus thymidine kinase (11), the dopamine receptor (12), or the Na/I symporter (NIS; 13, 14) and their corresponding positron-emitting markers. In the case of NIS, we have recently validated the quantitative nature of the images reconstructed after PET scanning (14).

In the present report, we have generated recombinant adenoviruses in which promoter fragments from either the hTERT or hTR genes drive the expression of the human NIS reporter gene. After injection of these recombinant adenoviruses (i.v. or intratumoral), followed by radiotracer iodide administration, the pattern of transgene expression was determined by PET scanning and by more classical postmortem bio-distribution studies. Both formulations provide selectivity of expression to cancer cells, and the study highlights the differences between the activities of the two promoters.

MATERIALS AND METHODS

Adenoviruses. The adenovirus Ad-Luciferase (immediate early cytomegalovirus promoter driving the expression of the luciferase cDNA) was a gift of Dr. P. Martin-Duque (Madrid, Spain). Ad-hNIS (immediate early cytomegalovirus promoter driving the expression of the human NIS cDNA) was described previously (15). To generate recombinant adenoviruses in which the telomerase promoter fragments drive the expression of the NIS cDNA, the human NIS cDNA (isolated by EcoRI digestion of FL2-hNIS/pCDNA3; Ref. 16) was inserted into the EcoRI site of pBlueScript to create pSSS1. The bovine growth hormone polyadenylation signal from pGv20 (17) was then inserted into the Spel/NorI sites of pSSS1 to create pSSS4. The NIS cDNA with the polyadenylation signal were then subcloned into the NorI/XhoI sites of pTGW7/pShuttle (Ref. 18) in which the HindIII site has been destroyed to create pTGW8. A promoter fragment of the hTERT gene (~190 to +54) from pG3shTERT-24/25 (5) or a promoter fragment of the hTR gene (~867 to +69) from pGL3Prom867 (5) was then introduced into the XhoI/HindIII sites of pTGW8. The production, purification, and titration of the recombinant adenoviruses Ad-hTERT-NIS or Ad-hTR-NIS were performed as described previously (14, 15, 18).

Cell Lines. PancO2 pancreatic carcinoma and A2780 ovarian carcinoma cell lines were grown in DMEM supplemented with 10% fetal bovine serum (Autogen Bioclear, Calne, United Kingdom), as described previously (5, 13). Animals. Six to eight week-old female C57Bl/6 mice or BALB/c nu/nu mice were obtained from Harlan (Oxfordshire, United Kingdom) and kept in a germ-free environment with irradiated food and acidified water ad libitum. Experiments were conducted after appropriate ethical approval and licensing was obtained in accordance with the United Kingdom “Guidance on the operation of animals (Scientific Procedure) Act 1986” (Her Majesty’s Stationery Office, London, United Kingdom, 1990). Tumor seeding, as well as adenovirus injections were performed as described previously (9, 13).

PET Scanning. Na124I was obtained from Hammersmith Imanet (London, United Kingdom). PET scanning was performed with a quad-HIDAC scanner as described previously (13, 14). A fraction of the dose solution was placed in the field of view to enable normalization of tissue data to injected dose. PET images were reconstructed as described previously (14). After the scan, mice
were sacrificed within 1–2 min by cardiac puncture, and various tissues were retrieved for postmortem gamma counting. In experiments that did not involve PET scanning, Na$^{125}$I was injected instead of Na$^{124}$I, as described previously (13, 14). Both imaging and postmortem bio-distribution data were expressed as a percentage of the injected dose per gram of tissue weight (%ID/g). Liver tissue was also fixed in 4% formaldehyde in PBS for immunohistochemistry.

Immunocytochemistry. Formaldehyde-fixed tissues were embedded in paraffin, sections cut and mounted on positively charged slides. Immunohistochemistry for NIS and adenoviral hexon protein was performed as described previously (15).

RESULTS

Imaging of the Pattern of Transgene Expression upon Systemic Injection of the Adenoviruses. Recombinant adenoviruses are known to infect a limited set of organs including the adrenal glands and the spleen, but the liver is recognized to be the dominant target after systemic injection in mice (13, 15). Because of the close proximity of these organs, PET imaging of transgene expression in mice, after systemic injection of recombinant adenovirus, can only provide quantitative information on the level of gene expression in the liver.

Systemic injection of Ad-Luciferase (Fig. 1A) led to a pattern of NIS expression similar to that obtained when PBS is injected (13), with iodide accumulation in the stomach and the thyroid gland (organs known to express the endogenous mouse NIS gene) and in the bladder, as a result of iodide excretion in the urine. When Ad-hNIS was injected, a strong, additional signal was observed in the upper abdomen, corresponding to the liver (Fig. 1B). Systemic injection of Ad-hTERT-NIS (Fig. 1, C1 and C2) produced a pattern of iodide accumulation identical in the four animals analyzed and similar to that observed when Ad-Luciferase was injected (Fig. 1A). Given the high sensitivity of PET analysis, these data suggest that the hTERT promoter is not active in liver cells.

Tail-vein injection of Ad-hTR-NIS followed by PET scanning produced the following two types of results: in three of the experi-
mental animals, absence of liver-signal was observed (Fig. 1D1), whereas a low level liver-signal was detected in two of them, as illustrated in Fig. 1D2. The intensity of the signal observed in these two animals was less than half of that obtained when Ad-hNIS was injected. Altogether, these data suggest that the hTR promoter has only a weak activity in hepatocytes.

**Postmortem Biodistribution of Transgene Expression.** After scanning, the animals were sacrificed, and the radioactive content of various biopsies was determined by direct \( ^{3}	ext{H} \)-counting. For animals receiving injection with Ad-hTERT-NIS, no signal above that of PBS-injected animals were observed in the liver, the adrenal glands and the spleen, suggesting that \( hTERT \) promoter is inactive in these tissues (Fig. 2). Ad-hTR-NIS injection led to iodide accumulation in the adrenal glands, the liver, and the spleen. However, this signal was consistently very weak. In addition, no iodide accumulation above that of PBS-injected animals were observed in the plasma, pancreas, lungs, hearts, large and small bowels, kidneys, and brains of animals systemically injected with either virus (Fig. 2).

**Immunohistochemistry.** To confirm that virus did indeed reach the liver, antibody staining for viral hexon protein was carried out. Fig. 3 shows immunohistochemical sections of livers of mice treated with Ad-Luciferase (A and B), Ad-hNIS (C and D), Ad-hTERT-NIS (E and F), and Ad-hTR-NIS (G and H). The immunoreactivities of the hexon (A, C, E, and G) and NIS protein (B, D, F, and H) were examined as an indicator of adenovirus presence/infection in the liver (hexon) and as a marker of promoter activity (NIS). Fig. 3 (A, C, E, and G) demonstrates that hexon-specific staining can be detected in all of the sections, confirming that all of the recombinant adenoviruses
were capable of infecting liver cells. No immunoreactivity was observed in liver sections of mice receiving injection with saline buffer (not shown). By contrast, NIS-specific signal was only detected in liver sections of animals receiving injection with Ad-hNIS (arrows in Fig. 3D). Therefore, immunohistochemical analysis suggests that both telomerase promoters are inactive or have a very low activity below the detection limits of the technique in liver cells. Altogether (Fig. 1, 2, 3), these data demonstrate that, in the context of a recombinant adenovirus, the hTERT promoter is inactive in all of the mouse tissues tested whereas the hTR promoter allows a low level of transgene expression in the adrenal gland, the liver, and the spleen.

Transgene Expression after Injection of the Delivery Vector in PancO2 Tumors. To establish the levels of gene expression allowed by the telomerase promoters in experimental tumors, the murine pancreatic carcinoma PancO2 cells were injected s.c. into syngeneic C57/B6 mice. The resulting PancO2 tumors were injected with either PBS, Ad-hTERT-NIS, Ad-hTR-NIS, or Ad-hNIS. Intratumoral injection of Ad-hTERT-NIS did not result in increased iodide accumulation compared with PBS-injected tumors (Fig. 4), indicating a lack of activity of the hTERT promoter in PancO2 cells in vivo. By contrast, intratumoral injection of Ad-hTR-NIS led to an accumulation of iodide to a level slightly higher but not significantly different (\(P = 0.096\)) from that obtained when Ad-hNIS was injected, suggest-
In the present study, we demonstrate formally that two promoter fragments from the hTR and hTERT genes can provide cancer-selective expression of the transgene when incorporated in a replication-deficient, recombinant adenoviral vector. In addition, the activity of these promoters can be visualized by PET imaging using NIS as a reporter gene. This methodology provides a noninvasive way to determine the magnitude as well as the location of gene expression. This is illustrated in Fig. 1 where individual variations in the efficiency of adenoviral infection can be observed and in Fig. 5 where heterogeneity of expression within a tumor injected with an adenovirus can be visualized in situ on live animals. Given that this methodology is directly scalable to humans, our results imply that imaging gene expression using the NIS PET reporter gene could be used to assess gene delivery and promoter activity in gene therapy clinical trials. These studies would involve a gene delivery vector encoding both a therapeutic transgene and NIS as a reporter gene. From the various coexpression approaches described thus far (reviewed in Ref. 19), the bicistronic, dual promoter, or bidirectional transcriptional approaches appear to be the most adapted to the NIS as a reporter gene. In addition, the NIS gene itself has been proposed as a therapeutic transgene through its ability to concentrate $^{131}$I in cancer cells (20–22). This dual potential may lead to gene therapy-mediated individualized radiotherapy.

Both the hTR and hTERT promoter appear to be good candidates for safe and specific use in gene therapy vectors, but differences have been highlighted. The hTERT promoter appears inactive in normal tissues infectable by systemic injection of recombinant adenovirus, and its activity in cancer cells in vivo is weak (Figs. 5 and 6) and restricted to certain cancer cells, as demonstrated by its lack of activity in PancO2 cells (Fig. 4). These data are consistent with our recent comparison of the hTR and hTERT promoters in a range of cell types (9). These characteristics suggest that this promoter could be used to drive the expression of transgenes that do not rely on high levels of gene expression to exert their therapeutic action. Examples of such transgenes can be found in the literature; e.g., adenoviruses encoding Bax and/or TRAIL driven by the hTERT promoter were reported to induce significant tumor regression in preclinical models as single agents or in combination (7, 8). More recently, a hTERT promoter fragment was used to drive the expression of the EIA gene in adenoviral vectors (23–27).

Turning to the hTR promoter, the basal level of radio-iodide accumulated in the spleen, adrenal glands, and the liver in PBS-injected animals (Fig. 2) was below plasma levels, suggesting that there is an overall exclusion of iodide from these organs. Systemic injection of Ad-hTR-NIS increased the radioactive content of these organs to levels observed in the plasma (Fig. 2), suggesting that the hTR promoter was allowing a less restricted pattern of gene expression than the hTERT promoter. However, this level of expression is low and much weaker than that obtained after intra-tumoral injection of Ad-hTR-NIS (Figs. 1, 2, 5, and 6). This differential level of gene expression between normal tissues and tumor cells highlights the potential applications of the hTR promoter. Because expression is detectable but low in normal tissues, the hTR promoter could be used to drive the expression of transgenes that do rely on high levels of gene expression to exert their therapeutic action, for example, pro-drug activating enzymes. In this context, an adenovirus in which the hTR-promoter drives the expression of nitroreductase (an enzyme capable of metabolising the pro-drug CB1954) has already shown therapeutic effects in preclinical studies (5, 9).

Taking into account the clear differentials in the levels of hTR and hTERT gene expression between normal and cancer cells in human biopsy samples (28, 29) and that mice have higher levels of telomerase than humans in all their adult tissues (30), any expression seen in these normal tissues in mice may well be exaggerated in comparison to humans. Therefore, the data presented in this report provide strong evidence for the cancer-cell selectivity of both telomerase promoters. In addition, we propose that a methodology based on minimally invasive, PET imaging of the NIS reporter gene could be applied to measure the activity of these promoters in humans, providing unique information on the pattern of gene transfer and transgene expression in patients.
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