Telomerase in alveolar epithelial development and repair

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Telomerase expression and activity were examined in the developing lung and in the adult lung during repair after injury. Both whole lung tissue and primary cultures of type 2 alveolar epithelial cells (AEC2) isolated from fetal and adult rodents were analyzed for 1) telomerase expression by immunohistochemistry and 2) telomerase activity with a telomerase repeat amplification protocol. We found that telomerase was expressed in a temporally regulated manner in fetal lung through the late stages of gestation, with peak expression just before birth. Expression persisted for a brief period in neonates, then decreased to nearly undetectable levels by postnatal day 9. Telomerase expression and activity were reinduced in normally quiescent adult lung by in vivo treatment with hyperoxia. In populations of AEC2 isolated from both developing and repairing lungs, telomerase expression and activity showed a strong correlation with the proliferation marker proliferating cell nuclear antigen. It has been suggested that telomerase expression and activity are hallmarks of stem or progenitor cells. Our observations suggest that a telomerase-positive subpopulation is present within the general AEC2 population. Telomerase may act as a marker for the proliferative status of this subpopulation.

Type 2 alveolar epithelial cells (AEC2) have been designated the primary progenitor cells of the alveolar epithelium (26). In the embryo, AEC2 arise from multipotent stem cells that line the primitive respiratory tract. These primitive proliferative embryonic epithelial precursors coexpress several markers including surfactant protein (SP)-A, SP-C, Clara cell 10-kDa protein (CC-10), and calcitonin gene-related peptide (CGRP), which are subsequently expressed in separate differentiated lineages including AEC2, Clara cells, and pulmonary neuroendocrine cells in the mature fetus and in the adult (32). At late gestation, the AEC lineage becomes restricted such that only type 1 alveolar epithelial cells (AEC1) and AEC2 are produced (19). AEC2 manufacture surfactant and can differentiate as required into AEC1 (27). AEC1 are terminally differentiated, incapable of dividing, and perform the necessary lung function of gas exchange. However, the ability to divide must be retained by a subpopulation within the lung alveolar epithelium throughout the lifetime of any animal to replace damaged cells (1, 7). This stem or progenitor cell function has been ascribed to AEC2.

A recent study (2) has shown that the ability of a cell to divide over an indefinite life span may require the expression of telomerase, a ribonucleoprotein that stabilizes the telomeres of chromosomes in actively growing cells. Telomerase contains structural features and activity similar to those of reverse transcriptases such that the catalytic subunit of the enzyme has been termed telomerase reverse transcriptase (TERT). Its structure and function are highly conserved among species. Human telomerase and its mouse ortholog, hTERT and mTERT, respectively, have recently been cloned and characterized (8, 12, 13, 15, 20, 23). In cells undergoing terminal differentiation, telomerase activity is downregulated, and chromosomal telomeres become progressively shorter (9). Multiple studies (9, 18, 19) have shown that in normal adult tissues, telomere length and telomerase activity appear to correlate well with the differentiation stage of a cell as well as with its potential to act as a stem cell on appropriate stimulation.

The self-renewing population of progenitor cells found in most tissues have been termed stem cells. Telomerase expression correlates with self-renewal potential in many cell types including epithelial cells (21, 33). Unlike tumor cells, stem cells are not immortal and show decreasing telomere length with increasing age (22, 31). However, telomerase may regulate self-renewal capacity by reducing the rate at which telomeres shorten. Understanding the function of telomerase in mediating self-renewal and survival of alveolar...
epithelial stem cells would be a major advance in AEC biology because a putative alveolar epithelial stem or progenitor cell could be the main source of epithelial expansion during development, of epithelial repair after injury, and, possibly, of alveolar adenocarcinoma.

Our current data indicate that a subpopulation of lung epithelial cells expresses telomerase throughout embryonic development and for a short time after birth. In the mouse, telomerase expression falls to nearly undetectable levels by day 9 after birth and remains at very low levels in the adult lung. However, this expression can be reinduced during the repair phase after lung injury. By examination of both whole lung and primary cultures of adult rat AEC2, we show that telomerase expression and activity increase in AEC during the proliferative phase of recovery from hyperoxia. These data may indicate that a hyperoxia-resistant subpopulation of telomerase-positive alveolar epithelial stem or progenitor cells is expanded in response to injury.

METHODS

Preparation of lung tissue and analysis by immunohistochemistry. Analysis of mTERT expression was performed on lung sections from embryonic and neonatal mice and on sections from control and hyperoxia-treated adult rats. Tissues were fixed in 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin according to the method described by Tesarollo and Parada (28). Tissues were sectioned, rehydrated, and subjected to immunostaining with a mouse and rat cross-reactive antibody to hTERT (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-positive cells were detected with reagents from the Histostain Plus kit from Zymed, with aminoethylcarbazole as the chromagen. Sections were observed and photographed with an Olympus light microscope. Immunocytochemical analysis of cultured cells followed essentially the same protocol; cells were fixed for 15 min in 4% paraformaldehyde in PBS, then stained according to manufacturer’s instructions with the Zymed kit. The proliferating cell nuclear antigen (PCNA) antibody used in this experiment was from Santa Cruz Biotechnology.

Telomerase repeat amplification protocol assay. Sample preparation and telomerase repeat amplification protocol (TRAP) assays were performed according to the TRAP-EZE protocol (Oncor). Briefly, at least 10^6 cells/sample were lysed in 1× 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer. The lysate was clarified by centrifugation, and protein content was measured with a modified Coomassie binding reagent from Bio-Rad. To assay telomerase activity, samples were incubated with a [γ-32P]dATP end-labeled telomerase-specific primer at 30°C for 30 min for telomere primer extension. The telomerase products were then amplified by 30 rounds of two-step PCR (94°C for 30 s and 60°C for 30 s). The samples were subjected to 12.5% nondenaturing PAGE in 0.5× TBE buffer (45 mM Tris-borate and 1 mM EDTA) for 1 h at 500 V. Gels were dried and exposed to X-ray film to visualize the telomerase products. Each assay included a positive control in the form of lysate from telomerase-positive A549 lung adenocarcinoma cells as well as a PCR internal amplification control provided by Oncor and a PCR contamination control lane consisting of all sample elements with the exception of cell lysate. All cell samples were individually controlled for non-specific PCR products by inclusion of a heat inactivation control for which identical aliquots of each sample were incubated at 85°C for 10 min to inactivate telomerase.

Hyperoxia treatment and adult and fetal AEC2 culture. Adult male Sprague-Dawley rats were exposed to short-term hyperoxia as described previously (5). Briefly, rats were placed in a 90×42×38-cm Plexiglas chamber and exposed to humidified >90% oxygen for 48 h and then allowed to recover in room air for 48 h. Control rats were kept in room air during the treatment period. At the end of the exposure or recovery period, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium. After complete exsanguination by normal saline perfusion via the pulmonary artery, lungs were lavaged to remove macrophages and then subjected to elastase digestion for isolation of AEC2. Differential adherence on IgG plates was used to eliminate non-AEC2 from the preparation (6). Embryonic day 21 (E21) fetal (saccular stage) rat AEC2 were obtained by trypsin digestion of whole lungs and differential plating according to the method of Jassal et al. (14). Timed-pregnant animals were euthanized by chloroform inrelation, and the fetuses were weighed to confirm gestational age. For both fetal and adult AEC2 cultures, cells were plated at 2×10^5 cells/cm^2 in DMEM with 10% FBS plus antibiotics for 24 h and then were harvested by trypsinization for TRAP assay preparation or fixed in situ for immunohistochemical analysis. Immunostaining of attached cells isolated by these methods with an anti-SP-C antibody confirmed that >95% of the attached cells were SP-C-positive AEC2 (5).

RESULTS

Telomerase expression is restricted to a subpopulation of mouse lung epithelial cells through embryonic development and is downregulated after birth. Lung sections from staged mouse embryos were fixed, paraaffin embedded, sectioned, and then immunostained with an antibody raised against hTERT, which cross-reacts with both mouse and rat TERT. Whole lungs were obtained from embryos on E18 (Fig. 1) and from neonates 1 h postbirth (day 0) and 2, 4, 6, and 9 days after birth. We found scattered epithelial and mesenchymal staining in lungs of mouse embryos from E18 through postbirth day 6. Epithelial expression appeared strongest at E18 through the day of birth, with staining confined to individual cells. Expression appeared to peak at this time and then declined over the next 9 days. During this period, the generalized expression pattern became restricted to discrete patches near the external surface of the lung (Fig. 1, D6). By postnatal day 9, telomerase expression was almost undetectable. A similar lack of mTERT expression was observed by immunostaining adult mouse lung epithelium (data not shown).

Telomerase expression in adult lung is induced during the repair phase after hyperoxic injury. Previous studies by our laboratory (4, 5) showed that exposure of animals to hyperoxia induces a proliferative response in normally quiescent lung tissue as part of a process of repair. To determine if reinduction of telomerase expression was a part of this process, fixed, paraffin-embedded sections were obtained from the lungs of adult rats that had been treated with hyperoxia for 48 h and then allowed to recover in room air for various periods of time. Lung sections from age- and weight-
matched animals that breathed room air throughout the treatment and recovery periods were used as controls. Sections were immunostained with the same anti-TERT antibody as described for embryonic and neonatal mouse lung sections. Our analysis showed that, as with adult mice, negligible TERT expression could be detected in control adult rat lung epithelium (Fig. 2, top). In contrast, TERT expression increased dramatically in the lung tissue of animals subjected to hyperoxia for 48 h and then allowed to recover in room air for 48 h (Fig. 2, bottom). Although scattered TERT expression was observed at 0 and 120 h of recovery after 48 h of hyperoxia treatment (data not shown), peak telomerase expression occurred during the period 48 h after treatment. Previous studies by Buckley et al. (4) and Bui et al. (5) showed that this is also the time period in which maximum proliferation of AEC2 after injury occurs. These data suggest that activation of telomerase expression may coincide with proliferation and activation of a stem or progenitor cell population that participates in repopulation of the damaged lung epithelium during recovery from injury. Alternatively, the high percentage of telomerase-positive cells observed during the repair phase may represent a hyperoxia-resistant population enriched by the loss of hyperoxia-sensitive cells during the injury period. Further experiments will be required to differentiate between these two possibilities or to determine if the telomerase expression profile of lung tissue after injury is due to a combination of both phenomena.

**Telomerase expression in fetal and adult AEC is correlated with proliferative status.** In situ immunostaining of whole lung tissue as described in Preparation of lung tissue and analysis by immunohistochemistry indicated that particular cells in developing and repairing lungs expressed telomerase, although the identity of those cells could not be determined. It has been postulated that AEC2 serve as the source of the reepithelialization in repairing lungs and perform the same function during the later stages of development. We speculated that cells performing these functions would, by necessity, express telomerase because they would be undergoing multiple rounds of cell division during both development and the repair process. AEC2 were isolated from E21 rat embryos according to the method of Jassal et al. (14). Fetal rat lungs are at the saccular stage of development within this time period, and the rat E21 stage was chosen to compare with the mouse E18.5 stage in which, by immunostaining, high mTERT expression was observed. AEC2 from adult
rats subjected to a hyperoxic environment for 48 h and then allowed to recover in room air for 48 h as well as control animals that breathed room air for the entire period were also isolated with the method described by Dobbs et al. (6). Both adult and fetal cells were plated on plastic in complete medium for 24 h. At that time, duplicate cultures were analyzed for telomerase and PCNA expression by immunostaining. The same anti-telomerase antibody and an adaptation of the protocol used for tissue section immunocytochemistry as de-
scribed in Fig. 1 were used for this purpose. The data obtained by these analyses showed that expression of telomerase was high in the majority of E21 rat AEC, although this expression was not completely uniform.

A similar pattern of expression was observed with the use of an antibody to the proliferation-specific protein PCNA (Fig. 3, B and C). In contrast, telomerase expression in the adult control sample was observed in very few cells (Fig. 3E). Surprisingly, a very low level of PCNA expression could also be detected in these cells, perhaps indicating a transient proliferative response to isolation and culture (Fig. 3F). Exposure to and recovery from hyperoxia in vivo resulted in reexpression of telomerase at high levels in the adult population in a pattern similar to that observed in the fetal population in that expression levels varied from cell to cell (Fig. 3H). These cells also exhibited a uniform increase in PCNA expression, indicating a strong proliferative profile (Fig. 3I). Thus telomerase expression was confined in the adult AEC to a portion (albeit, in response to hyperoxia, a large one) of the total population. Specificity of expression for the telomerase antibody was confirmed by use of normal rabbit serum in place of primary antibody (Fig. 3, A, D, and G).

Telomerase activity is observed in fetal AEC2 and can be reinduced in adult AEC2 after hyperoxic injury.

To determine if the TERT expression observed in fetal and adult lung tissues could be correlated with telomerase activity in lung epithelial cells, we harvested a portion of the primary cultures of AEC2 isolated from fetal and adult rats and analyzed them for endogenous telomerase activity by use of the PCR-based TRAP assay. For each sample, radioactively labeled telomerase end products (telomeric repeats; TRs) were subjected to TBE-PAGE, and the results were detected by radiography. The number of TRs produced by each sample was counted to give a relative estimate of the telomerase activity contained within the sample, each of which contained 80 ng of each cell lysate. With a ratio of 10 ng protein/cell, the telomerase activity observed in each sample was taken to correspond to the activity present in approximately eight AEC2. Although these isolated AEC2 populations are, by SP-C staining, 95% pure (5), the minor fibroblast contamination that is still present in primary cultures could account for some of the observed telomerase activity. Therefore, the number of cell equivalents used for each assay was kept at this minimum level to ensure that the sample contained lysate enriched to a high degree with epithelial cell components and that fibroblast contribution to the assay remained negligible.

The results of the TRAP assay showed that telomerase activity could be correlated with telomerase expression as assayed by immunostaining during both development and injury repair. Although the level of telomerase activity in the E21 fetal AEC2 was quite high (Fig. 4A, lane 6), the level in the same number of cells obtained from a 6-wk-old adult male rat was much lower (Fig. 4A, lane 2). Interestingly, AEC2 obtained from adult animals that had been exposed to a hyperoxic environment showed a significant increase in

Fig. 2. Telomerase expression in hyperoxic lung injury and repair. Whole lungs from adult rats subjected to hyperoxia for 48 h and then allowed to recover for 48 h in room air were obtained along with lungs from control animals that had breathed room air throughout the treatment period. Lung tissue was fixed, paraffin embedded, sectioned, and then subjected to immunohistochemical analysis for rat (r) TERT expression. Control animals exhibited almost no TERT expression in lung epithelium (top). However, the hyperoxia-treated lungs showed a marked increase in rTERT expression (bottom). Original magnification, ×200.
telomerase activity (Fig. 4A, lane 4). This result was reproducible, and Fig. 4B shows a quantitation of the TRAP assays performed on a number of animals (values are means ± SD; n = 3 fetal and 6 adult animals). The average number of TRs produced by each type of sample was correlated to ladder bands by the formula TR = n + 3, where n is the number of bands in each ladder and TR is taken in this assay as a reflection of relative telomerase activity. By this calculation, the fetal sample produced the largest average number of TRs, 24. The sample from the control animals contained the fewest average number of TRs, five, as would be expected from cells obtained from highly differentiated tissue. The number of repeats produced by lysates of cells from animals treated with oxygen showed a consistent increase, reflecting increased telomerase activity. The average number of TRs produced by hyperoxic adult AEC2 was 18. With Student’s t-test, the differences in the TRs between control and hyperoxic (P < 0.005) and between control adult and fetal (P < 0.001) cells were determined to be significant.

DISCUSSION

The data presented here demonstrate that telomerase, the polymerase responsible for telomere maintenance and extended cellular life span, is expressed in developing rodent lung and then downregulated after birth. It has been established by Greenberg et al. (8) that mTERT mRNA expression peaks in the whole embryo at midgestation (E9.5 through E15.5) during mouse development. Interestingly, the mTERT transcript is maintained at a very low level compared with that of a housekeeping gene such as actin. However, expression is broadly distributed through many organs including the lung. The transcript level in the lung of newborn animals is intermediate between the high levels observed in organs with proliferative indexes (intestine, testes) and the low levels in organs composed of less proliferative tissues (brain, heart). This pattern is maintained in adult animals, indicating that whole mouse lung may require a certain basal level of telomerase expression for proper function. However, the identity of the telomerase-expressing cells in the lung was not described by Greenberg et al., who used whole lung lysates as a source of TERT mRNA. Here, we report that the expression of mTERT as assayed by immunostaining of sections of lungs harvested from staged mouse embryos and neonates, is restricted to a subpopulation of cells within the alveolar epithelium. In mice, expression levels peak at E18.5, just before birth, and then decrease over the period of alveolarization that occurs 4–6 days after birth. By 9 days after birth, expression of the mTERT protein is almost undetectable. As expected, mTERT expression is also low in adult lungs from normal animals.

Drawing analogies directly from these studies to human lung development raises certain caveats, however, because lung development in humans differs from that observed in rodents. For example, alveolarization occurs in humans before birth, whereas in both rats and mice, it is a postnatal process (29). In addition, several experiments have demonstrated that telomere maintenance in the murine system may be quite different from that in humans. The telomerase RNA component knockout mouse exhibits no aberrant phenotype within the first six generations, perhaps because of the fact that the telomeres of mouse chromosomes are much longer than their human counterparts (3). In addition, telomerase activation does not appear to be
Fig. 4. Telomerase activity in fetal and adult AEC2. A: AEC2 were isolated by standard methods from fetal rats (E21), adult rats subjected to hyperoxia for 48 h (no recovery period), and control adult rats that breathed room air for the same period. Cells were cultured for 24 h in DMEM-10% fetal bovine serum before being harvested. Lysates were prepared and protein quantities measured such that 80 ng of protein from each lysate were included in each sample. Duplicate samples were heat treated to provide a control for heat-tolerant PCR contaminants. These samples were loaded into lanes 1, 3, and 5. Lanes 2, 4, and 6 contain the results of the telomerase repeat amplification protocol (TRAP) assay for the untreated samples. Lanes 1 and 2, 80 ng each of heat-treated and untreated control adult rat AEC2 lysate, respectively; lanes 3 and 4, 80 ng each of heat-treated and untreated hyperoxia-treated adult rat AEC2 lysate, respectively; lanes 5 and 6, 80 ng each of heat-treated and untreated fetal rat AEC2 lysate, respectively. E: no. of telomeric repeats (TRs) present in each sample was correlated to ladder bands by the formula TR = n + 3, where n is the no. of bands in each ladder, and TR is taken in this assay as a reflection of relative telomerase activity. By this calculation, the fetal samples produced the largest number of TRs, the average being 24. The samples from the control animals contained the fewest number (average TRs = 5). Reflecting increased telomerase activity, the number of TRs was increased by treatment of adult animals with oxygen (average TRs = 18). For the fetal TR average, n = 3, whereas for the adult averages, n = 6. Values are means ± SD. With Student’s t-test, the differences between control and hyperoxia (+P < 0.005) and between control adult and fetal (;++P < 0.001) cells were significant.

required for tumor induction in primary cultures of cells of murine origin, although it has been demonstrated to be essential for the conversion of normal human cells to a cancerous phenotype (11). This difference may be due to the fact that a low level of telomerase expression has been observed in adult mouse somatic cells (16), although our observations with immunostaining showed little evidence of this expression in the lung.

In adult rats, telomerase expression can be reactivated in lung epithelial cells in response to injury. Assays for both its expression and activity revealed that telomerase is induced in AEC2 during the recovery phase after injury. Changes in telomerase expression in adult cells have been documented in other tissues. In addition to altered expression in the development of cancer, telomerase expression is reinduced in several tissues as part of a normal physiological response to hormonal stimulus (25) or in response to injury (10, 13, 18, 24). It has long been speculated that after acute hyperoxic lung injury in the adult rat, a specialized population of AEC survive and regain the capacity to proliferate and repopulate the denuded, damaged alveolar epithelial surface (1, 30). If these cells depend on telomerase activity for the proper proliferative response, then it may be that telomerase could function in stem or progenitor cells as a mediator of survival as well as of self-renewal capacity.

We observed that in situ the percentage of cells that express telomerase is higher in the repairing adult lung than the percentage observed in developing lung tissue, although the percentages of positive cells in the isolated, AEC-enriched populations in culture are similar. This discrepancy may simply reflect that the percentage of AEC2 in the developing lung is much smaller than that observed in the adult lung and that isolation of an AEC-rich population pools together all the telomerase-positive lung cells from each source. The large number of telomerase-positive cells in the AEC population isolated from repairing lung could represent those cells that have repopulated the damaged tissue during the injury and recovery periods and that may soon exit the proliferative pool to take up AEC2-differentiated functions, although this has yet to be proved.

These data raise the question of whether telomerase expression in the repairing lung is simply a marker for proliferation and whether it is expressed more ubiquitously than would be expected for a stem cell population. This possibility has been addressed in other systems by Kolquist et al. (17), who showed that specific subsets of cells thought to have long-term proliferative capacity can be observed in normal quiescent tissues. Interestingly, this study, which analyzed human tissue with in situ hybridization of hTERT mRNA, showed no expression in normal lung, similar to our observation in mouse and rat lung tissues in which we rarely see positive nuclei. Indeed, under culture conditions, it appears that induction of telomerase expression parallels the proliferation profile as indicated by PCNA
expression, of each cell type examined. If telomerase does act simply as a marker for proliferation in AEC2, other markers that would allow differentiation between subpopulations of telomerase-positive cells would have to be determined before a true progenitor cell population could be isolated and used for functional studies.

It should be noted, however, that Kolquist et al. (17) demonstrated a difference in intensity in telomerase expression exhibited by normally proliferating tissue and neoplastic tissue and speculated that the level of telomerase expression may be the factor that distinguishes stem cells from other proliferating cells. In the current study, we note that the intensity level of telomerase expression in the fetal AEC population was consistently stronger than that observed in the adult population both in situ and in culture. In addition, telomerase expression in both fetal and hyperoxic rat AEC2 in culture shows variable intensity within each population, indicating that even within the large percentage of telomerase-positive cells, subpopulations may exist that could be differentiated on the basis of levels of telomerase expression. However, because no method exists at this time for following the fate of individual cells in the lung, it is impossible to determine when and how telomerase expression is induced and how long it persists in each individual cell.

Bui et al. (5) have previously shown that cyclins A and D and the cyclin-dependent kinase cdk2 are induced and activated in proliferative AEC after hyperoxia, suggesting that an AEC2 subpopulation is indeed activated to divide in response to injury. Buckley et al. (4) showed that a large proportion of AEC2 isolated from hyperoxia-treated animals undergo DNA damage and apoptosis when placed in primary culture on tissue culture plastic. Thus it is tempting to speculate that two diverse compartments exist within the AEC population. Whereas the majority of terminally differentiated AEC are apparently damaged beyond repair by hyperoxic injury and die in culture (4), there must be a subpopulation of proliferative alveolar epithelial stem or progenitor cells with the capacity to survive injury that is responsible for repopulating the damaged lung epithelium. Whether this action of alveolar stem or progenitor cells depends on telomerase expression remains to be determined.

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