High Levels of Telomere Dysfunction Bestow a Selective Disadvantage During the Progression of Human Oral Squamous Cell Carcinoma

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ABSTRACT

Human epithelial cells experience multiple barriers to cellular immortality in culture (mortality mechanisms 0, 1, and 2). Mortality mechanism 2 (M2) is termed crisis and involves telomere dysfunction due to lack of telomerase. However, proliferating normal keratinocytes in vivo can express telomerase, so it is unclear whether human squamous cell carcinomas (SCCs), which usually have high telomerase levels, develop from preexisting telomerase-positive precursors or by the activation of telomerase in telomerase-deficient somatic cells. We show that 6 of 29 oral SCCs show characteristics of M2 crisis in vivo, as indicated by a high anaphase bridge index (ABI), which is a good correlate of telomere dysfunction, and that 25 of 29 tumors possess some anaphase bridges. ABIs in excess of 0.2 in the primary tumor showed a decrease in the corresponding lymph node metastases. This suggests that high levels of telomere dysfunction (>0.2) and, by inference, M2 crisis bestow a selective disadvantage on SCCs during progression stages of the disease. Supporting this, SCCs with high levels of telomere dysfunction grow poorly in culture, and the ectopic expression of telomerase corrects this, together with other features of M2 crisis. Our data suggest that a substantial proportion of oral SCCs in vivo ultimately arise from telomerase-deficient keratinocytes rather than putative telomerase-proficient cells in the undifferentiated parts of the epithelium. Furthermore, the presence of significant levels of telomere dysfunction in a high proportion of SCCs at diagnosis but not in the normal epithelium implies that the therapeutic inhibition of telomerase should selectively compromise the growth of such tumors.

INTRODUCTION

Human cells, including keratinocytes, are known to exhibit a limited replicative life span that culminates in replicative senescence (1). In epithelial cells, including keratinocytes (2), replicative senescence is controlled firstly by the accumulation of p16INK4A (M0); Refs. 2-5 and then by the increased activity of p53 (2, 6, 7), probably as a consequence of telomeric attrition (M1; Refs. 8-10). M0 and M1 are biologically indistinguishable (8), and both are referred to as replicative senescence (8, 10). DNA tumor viruses abrogate the functions of the p53 and pRb axes, bypass replicative senescence (M0 and M1), and extend the proliferative life span of human somatic cells (4, 10). These cells continue to exhibit telomeric attrition and eventually enter crisis (M2; Refs. 2 and 10-13). In some instances, rare immortal variants emerge that have corrected telomere dysfunction, either by up-regulating telomerase (10-13) or, less commonly, by activating the recombination mechanism known as ALT (14).

There is now considerable evidence that M1 and M2 are bypassed by the ectopic expression of the catalytic subunit of telomerase, hTERT (4, 11, 12, 15-18). In human keratinocytes and other epithelial cells, however, the continued presence of M0 results in either senescence (4) or slowed proliferation (15, 17).

Most human cancers harbor mutations in the p53 and pRb pathways and are telomerase positive, suggesting that M0, M1, and M2 are sequentially bypassed as these cancers progress in vivo (10, 13, 14, 19, 20). The genetic analysis of in vivo squamous neoplasms (21-23) and their mortal and immortal counterparts (3, 6, 7, 24-26) supports this view, at least in the case of M0 and M1. However, evidence for a M2 barrier to human carcinoma development, as a result of inadequate telomerase function, is less convincing.

M2 crisis may not constitute an equally severe barrier to the immortalization of all cell types (13), and this view is supported by the observation that SV40-infected human keratinocytes emerge from M2 crisis at a much greater frequency than their fibroblast counterparts (27). Furthermore, a recent study has shown that a high level of telomere dysfunction, as assessed by ABIs, can occur in human cancer, even in the presence of detectable telomerase activity (28), suggesting that these telomerase-positive cancers may tolerate such dysfunction. However, it was unclear from this study whether the anaphase bridges resulted from inadequate telomerase. Telomerase activity is essential for the immortality of human SCC and other cancer cells in vitro (29, 30) and for the formation of mouse squamous tumors in vivo (31). Human tumors generally have shorter average TRF lengths than the corresponding normal tissue (32, 33). Therefore, because TRF lengths are not maintained, it is often argued that telomerase is deficient in normal cells and in the early stages of cancer and is subsequently reactivated to restore telomere function (10, 13). However, there are other plausible explanations for the shorter TRF lengths in human carcinomas (32). Furthermore, the proliferative compartments of many epithelia express telomerase (34-38), leading to the suggestion that human cancers may, instead, arise from preexisting telomerase-positive cells that expand during tumor progression, perhaps as a consequence of blocked terminal differentiation (39). In such a scenario, the selection for p16INK4A and p53-deficient cells might be achieved as a consequence of oncogene activation (40) or DNA damage (41, 42). Therefore, in human carcinomas, it is not completely clear whether telomerase is reactivated by chromosomal alterations of the target cells to bypass M2 (10, 13, 26) or, alternatively, whether preexisting telomerase-positive cells expand during tumor progression for other reasons (39). Also, if telomerase levels are inadequate to sustain telomere function in developing human carcinomas, it is still not clear how much this compromises the growth rate of the tumor (13, 28).

The reactivation hypothesis would predict the existence of neoplastic human cells, which have bypassed M0 and M1 but not M2. Moreover, these cells would also be predicted to enter M2 crisis when allowed to proliferate further in vitro. For the first time, we have identified and characterized such cells and demonstrate that they can

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The abbreviations used are: M0, mortality mechanism 0; M1, mortality mechanism 1; M2, mortality mechanism 2; ABI, anaphase bridge index; SCC, squamous cell carcinoma; OSCC, oral SCC; ALT, alternative lengthening of telomeres; pRb, retinoblastoma protein; TRF, telomere restriction fragment; FBS, fetal bovine serum; TRAP, telomeric repeat amplification protocol; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; MPD, mean population doubling; hTERT, human telomerase reverse transcriptase.

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be immortalized by the ectopic expression of hTERT. Furthermore, we demonstrate the presence of cells with similar properties in a subset of in vivo SCCs and show that they possess a selective disadvantage during in vivo tumor progression.

MATERIALS AND METHODS

Cell Cultures. The isolation and establishment of the neoplastic keratinocyte cultures and cell lines used in this study have been described previously (6, 7). The keratinocytes were cultured using Swiss 3T3 feeder layers in DMEM supplemented with 10% (v/v) FBS and 0.4 μg/ml hydrocortisone, as described previously (6). This medium formulation was arrived at by testing each BICR culture in the above plus four additional separate media at the point when the keratinocytes were released from explant cultures (see Ref. 5). The other media were: (a) flavin adenine dinucleotide medium supplemented with 10% (v/v) FBS, 0.4 μg/ml hydrocortisone, 0.5 μg/ml insulin, 5 μg/ml transferrin, 10⁻¹⁰ M cholera toxin, 1.8 × 10⁻⁴ M adenine, and 10 ng/ml epidermal growth factor (added at the first medium change); (b) DMEM, 20% FBS, for hydrocortisone, and cholera toxin; (c) DMEM, 5%, FBS, and hydrocortisone; and (d) DMEM, 2% FBS, and hydrocortisone. DMEM, 10% FBS, and hydrocortisone proved to be the best formulation for >80% of the BICR lines so tested, including all of the BICR lines used in the current study. The elimination of hydrocortisone from the media was severely detrimental to growth, as described previously for normal keratinocytes (1). All other cell lines were cultured in the same medium without the hydrocortisone, with the exception of 3T3 cells, for which bovine serum was used instead of FBS.

Immunocytochemistry. Briefly, 8-μm-thick paraffin sections were mounted on plain glass slides. After deparaffinization in xylene and rehydration in graded alcohols, the slides were placed in 1% hydrogen peroxide for 5 min to quench endogenous peroxidase. Citrate buffer (0.01 m, pH 6.0) was heated in a microwave pressure cooker at maximum power (850 W) for 15 min, and the slides were then added. The slides were heated in buffer for 4.5 min at full power under pressure. Sections were immediately cooled in PBS. Thereafter, all of the sections were treated with blocking antibody in PBS for 1 h at room temperature. Slides were incubated with the primary monoclonal antibody [P16INK4A (F+C), P53 (DO-1); both from Santa Cruz Biotechnology] in a dilution of 1:400 (P16INK4A) and 1:300 (P53) overnight at 4°C. The rest of the procedure (secondary antibody, avidin-biotin complex, and color development) was performed using the mouse avidin-biotin complex method kit and 3,3′-diaminobenzidine kit (both from Vector Laboratories Ltd). Sections were then counterstained with hematoxylin, dehydrated, and mounted in histomount (Hughes and Hughes Ltd.). The p16INK4A antibody used was raised against the COOH terminus of the p16INK4A protein (Santa Cruz Biotechnology). Positive controls for this antibody included normal cultured keratinocytes, SCC keratinocytes, and normal oral mucosa. Within the tumor sections, the presence of good nuclear staining of fibroblasts, endothelial cells, macrophages, and lymphocytes confirmed that antigen retrieval had worked. Negative controls were formalin-fixed cell lines BICR6 and BICR7, both of which possess homozygous deletions of the INK4A locus. The p53 protein was detected using the mouse monoclonal antibody DO-1. The negative control was tumor BICR6, which does not express detectable p53 protein, and the positive control was BICR5, which does express detectable p53 protein (24). Normal tonsil was used as a control for normal p53 levels. The primary antibody was also eliminated as an additional negative control for both the p16 and p53 antibodies.

Retrovirus Production and Ectopic Expression of hTERT. The retrovirus were produced by infecting the amphotropic packaging line Phoenix A with pBABeNeo or pBABeNeo retroviral constructs (kindly provided by Dr. H. Vaziri). SCC keratinocytes were infected when 80% confluent with 1.35 × 10⁴ infectious units/ml, in the presence of 4 μg/ml Polybrene. The SCC cells were plated with G418-resistant 3T3 cells and selected 48 h later with 150 (BICR7), 200 (BICR5 and BICR63), and 600 (BICR6) μg/ml G418 for 2–3 weeks. Pooled G418-resistant colonies were used in all experiments to avoid variation due to clonal heterogeneity of the population.

Telomerase Activity. Cellular extracts were prepared and assayed for telomerase activity using the TRAPEze kit (Intergen Co.), following the manufacturer’s instructions. Pilot experiments were initially carried out on a range of protein concentrations (0.2–2 μg) to determine the linear range of the assay for each cell line. Assays were carried out on 0.4 and 0.8 μg of protein extracts for each cell line. The PCR products were resolved on 10% non-denaturing polyacrylamide gel. Telomerase activity in the extracts was quantified by determining the ratio of the 36-bp internal standard to the telomerase ladder using the Bio-Rad molecular imager and Quant One imaging software package. Typical examples of TRAP assays are shown.

TRF Measurement. Genomic DNA was isolated from each of the cell lines (43), and 4 μg were double-digested with HindIII and RsaI (Roche Diagnostics) overnight at 37°C. The digests were resolved on a 1% TBE (0.05 m Tris base, 0.05 m boric acid, and 1 mM EDTA) pulse field gel (CHEF-DR II System; Bio-Rad) at 6 V/cm with an initial switch of 1 s and a final switch of 6 s for 16 h. The gel was transferred to Zeta-Probe GT membrane (Bio-Rad) by capillary action in 0.4 N NaOH for 16 h. The membrane was rinsed in distilled water, and the DNA was cross-linked to the membrane using a UV cross-linker (Stratagene). The blot was hybridized with a radiolabeled oligonucleotide probe (TTAGGG)₁₃ at 42°C and subsequently washed to a stringency of 3 × SSC/0.1% SDS at 42°C. The telomere lengths were measured as described previously (44).

Colony Forming Efficiency. Five hundred cells were plated in triplicate on 5-cm plates with 3 × 10⁴ Swiss 3T3 feeders and grown for 2–4 weeks. Cells were washed in PBS and then fixed in methanol and stained with 1% (w/v) rhodamine B. Colonies larger than 1 mm were counted and expressed as the total number of cells seeded. The size of individual colonies was estimated using the Scion Image (Scion Corp.) software package. Initially, a set area was selected, and a threshold was applied to account for background staining. The total number of pixels/dish was calculated and used to determine the total surface of the stained area. This was then divided by the number of colonies/dish to determine the average surface area of each colony.

BrdUrd Labeling Index. Cells (2.5 × 10⁴) were seeded with 1 × 10⁴ feeder cells, and 6-well glass chamber slides (Labtek; Nalge Nunc International Inc.) were used and incubated at 37°C for 1 week to allow formation of discrete colonies. The cells were incubated with 10 μM BrdUrd (Sigma Chemical Co., Poole, United Kingdom) for 48 h and processed as described previously (44). The nuclei were counterstained with Hoechst 33258 (0.05 μg/ml) to differentiate between feeders and keratinocytes (45) before mounting. Negative controls were set up for nonspecific background staining by replacing the BrdUrd with PBS during the 48-h incubation.

TUNEL Assay. Cells (2 × 10⁴) were seeded in 5-cm dishes with 3 × 10⁴ irradiated Swiss 3T3 feeders and incubated for 1 week at 37°C to allow discrete colonies to form. Cells were fixed for 10 min in 1% paraformaldehyde and washed 2 × 5 min in PBS. The cells were postfixed in precooled ethanol/acetic acid for 5 min at −20°C, drained, and washed as above. TUNEL assay was carried out using the Apoptag Fluorescein In Situ Apoptosis Detection kit (Intergen Co.) as indicated by the manufacturer. The cells were mounted in Vectashield/4,6-diamidino-2-phenylindole and visualized on a fluorescence microscope, and apoptotic cells were scored based on morphology and positive TUNEL staining. The keratinocytes were clearly distinguishable from the 3T3 cells (see above).

Dicentric Chromosome. Metaphase chromosome spreads were prepared using standard procedures and analyzed as reported previously (11, 12).

Anaphase Bridges. H&E-stained cultures or tissue sections were examined under a light microscope using ×100 magnification for anaphase bridges. The ABI was determined by dividing the number of anaphases with bridges by the total number of anaphases. Anaphase bridging was defined as reported previously (46). A total of 10–66 anaphases from 1–4 slides were examined by two investigators to obtain the ABI for each sample.

Statistical Analyses. To test the statistical significance of quantitative data, the Wilcoxon-Mann-Whitney nonparametric rank test was used when quantitative data were compared (47).

RESULTS

An OSCC, BICR7, Has Bypassed M0/M1 in Vivo. Keratinocyte cultures were derived from 23 head and neck SCCs. Thirteen of the cultures were immortal and included the three immortal controls used in this study (BICR6, BICR31, and BICR63). Nine cultures senesced, even in the best-known keratinocyte culture conditions (17). The BICR7 culture was not immortal, although it had bypassed M0 and
M1 as indicated by the lack of functional p16INK4A and p53 (Refs. 6 and 7; Table 1). We immunostained the original BICR7 tumor with p16INK4A- and p53-specific antibodies to confirm that M0 and M1 were bypassed in vivo (Fig. 1). It can clearly be seen that the group of BICR7 tumor cells in Fig. 1A is negative for p16INK4A immunostaining, (red arrows), apart from an infiltrating macrophage (black arrow), and this contrasts with the basal and immediate suprabasal staining pattern in normal tonsil (Fig. 1B). Similar groups of BICR7 tumor cells were positive for p53 immunostaining (Fig. 1C), whereas the expression in normal tonsil was low (Fig. 1D), and the BICR6 control section was negative (Fig. 1E). Immunoreactivity with p53 antibodies is not conclusive proof that the p53 pathway is compromised, but we had previously confirmed the presence of codon 151 Pro→His mutation, in both the BICR7 cell culture and the original BICR7 sample (24). The absence of p16INK4A in the BICR7 cell culture is consistent with the homozygous deletion of the INK4A locus in the BICR7 cell culture (3).

Ectopic Expression of hTERT Rescues the M2 Crisis Displayed by BICR7 in Vitro. The BICR7 culture had a slow growth rate immediately upon placement in vitro and totally ceased growth after 40–45 generations (20 MPDs). The high MPD cultures contained some colonies with a normal morphology, but the majority consisted of flat multinucleated abortive colonies (Fig. 2A). No telomerase activity was detected in early-passage cultures, but very low levels could be detected in later passages (Fig. 3).

To test whether the phenotypes observed in BICR7 were due to low levels of telomerase, we introduced the hTERT expression vector pBabest2 or neomycin-resistant control vector pBabeNeo into BICR7 cultures (designated 7TERT and 7NEO, respectively) and three immortal control lines by retroviral infection (18). Most of the colonies in the 7TERT cell line grew progressively, and the morphology of the cells resembled that of the immortal BICR31 cell line (Fig. 2, B and C). The growth rate of the hTERT-expressing culture was also increased (data not shown), and to date, the cells have completed >50 MPDs without showing any sign of senescence or crisis.

The introduction of hTERT into BICR7 increased telomerase activity in 7TERT cultures approximately 80–100-fold compared with the parental BICR7 and the 7Neo cell line and above the levels seen in the BICR63 immortal cell line (Fig. 3). In addition, the telomerase activities of all of the immortal control lines were also increased by the introduction of hTERT.

The effect of this increased telomerase activity on telomere length was examined by comparing the TRF lengths of the different cell lines (Fig. 4). The 7Neo cell line possesses a very short (3-kb) average TRF length, approximately 1–4 kb shorter than that of the immortal cell lines (Fig. 4). The introduction of hTERT increases the average TRF length to around 10 kb (7TERT), which is in excess of the length seen in pre-M0 keratinocytes (Ref. 15; Fig. 4), but in contrast, the introduction of the Neo cassette alone was without effect, arguing against a selection for BICR7 clones with longer telomeres. The TRF lengths of all of the immortal control lines were also increased to a similar size (9–12 kb). The very high molecular weight smear observed in BICR7, 7Neo, BICR63, and 63Neo is often seen in keratinocyte TRF blots. These bands show a similar migration pattern to 3T3 TRFs control lane. Because mouse TRFs are 10–25 times more abundant than the human keratinocyte lines in our study, a very small number of 3T3 cells remaining after EDTA treatment would be expected to produce a strong TRF signal.

To determine whether the ectopic expression of hTERT would bypass the M2 crisis observed in BICR7, we examined several mark-
physiological levels of the enzyme that they expressed (high cloning efficiencies to begin with, were unaffected by the sup-
BICR31 and BICR6, which have abundant telomerase activity and
displays some evidence of telomere dysfunction (see below). This
be immortal. However, it does have short average TRF lengths (4 kb)
possesses moderate levels of telomerase (Fig. 3) and is considered to
the ectopic expression of hTERT is not due to a nonspecific effect of
It also argues that the improvement in BICR7 growth sustained after
This important observation suggests that if a cell line has sufficient
to the culture dish, despite the fact that they express other markers of
A.

culture is one where less than 5% of the cells cycle in 48 h (48).
We next examined the level of apoptosis in the cell lines using the
We performed BrdUrd incorporation assays to confirm that the 7Neo cell line
incorporates BrdUrd in 48 h while displaying a slow increase in cell number. As expected, the introduction of hTERT did not affect the
rate of BrdUrd incorporation into BICR7 in 48 h because the rate was already in excess of 97% (data not shown), consistent with the cells
being in crisis and not senescent. Since the definition of a senescent
We carried out colony forming assays to compare the growth properties of each of the cell lines. Fig. 5A shows that the elevation of
telomerase levels in BICR7 largely corrected the poor cloning efficiency of the culture, raising it from 3% in the 7Neo cell line to 28%
in the 7TERT line ($P < 0.002$). In contrast, the control cell lines BICR31 and BICR6, which have abundant telomerase activity and
high cloning efficiencies to begin with, were unaffected by the sup-
rhapsiological levels of the enzyme that they expressed ($P > 0.2$). This
important observation suggests that if a cell line has sufficient
endogenous telomerase activity, then telomere function can be sus-
tained, even in the presence of TRF lengths as short as 4 kb (BICR6).
It also argues that the improvement in BICR7 growth sustained after
the ectopic expression of hTERT is not due to a nonspecific effect of
the transgene on cell viability or proliferation. The cell line BICR63
possesses moderate levels of telomerase (Fig. 3) and is considered to
be immortal. However, it does have short average TRF lengths (4 kb)
and displays some evidence of telomere dysfunction (see below). This
cell line did reveal a small, insignificant ($P > 0.1$) increase in cloning
efficiency upon the expression of hTERT, but a significant increase in
ery of crisis in the 7Neo, 7TERT, and immortal controls. We
performed BrdUrd incorporation assays to confirm that the 7Neo cell line
incorporates BrdUrd in 48 h while displaying a slow increase in cell number. As expected, the introduction of hTERT did not affect the
rate of BrdUrd incorporation into BICR7 in 48 h because the rate was already in excess of 97% (data not shown), consistent with the cells
being in crisis and not senescent. Since the definition of a senescent
culture is one where less than 5% of the cells cycle in 48 h (48).

We next examined the level of apoptosis in the cell lines using the
TUNEL assay. Ectopic expression of hTERT in BICR7 reduced the
percentage of TUNEL-positive cells from 5.8% to 1.8%, (Fig. 5B),
suggesting that the low cloning efficiency of the BICR7 culture is partly due to increased rates of apoptosis, possibly as a consequence of
telomere dysfunction. However, the 3-fold decrease in the number of
TUNEL-positive cells did not fully account for the 8-fold increase in
colony forming efficiency observed in BICR7 after the introduction
of hTERT. One possible explanation is the sensitivity of the TUNEL
assay. It has been documented that many human epithelial cell types
in M2 crisis express low levels of TUNEL staining while still attached
to the culture dish, despite the fact that they express other markers of
apoptosis (8), but it is also possible that telomere dysfunction triggers
death by mechanisms other than apoptosis.

**Increased Telomerase in BICR7 Corrects Telomere Dysfunction.** The above observations suggested that BICR7 was indeed in M2
 crisis and that this was due to inadequate telomerase levels, but it was still unclear whether the results were connected with correction of
telomere dysfunction or whether they were the result of other proposed effects of telomerase, such as the inhibition of apoptosis, for example (49). Although it has already been shown that the ectopic expression of telomerase can cooperate with p16/pRb and p53 deficiency to immortalize normal and neoplastic human keratinocytes (2, 4, 15), it has also been shown that the ectopic expression of hTERT can improve wound repair and facilitate carcinogenesis in mouse epidermis, where the telomeres are already very long and presumably fully functional (50).

To test the involvement of telomere function in the escape of
hTERT-expressing BICR7 cells from M2 crisis, we quantified the
number of dicentric chromosomes and anaphase bridges in BICR7
cells with and without hTERT because both dicentric chromosomes
and anaphase bridges have been shown to be good correlates of
telomere function *in vivo*. Fig. 6 shows that BICR7 displays high
levels of dicentric chromosomes (Fig. 6, A and B) and anaphase
bridges (Fig. 6, C and G; see also Fig. 6F, a normal anaphase), as well
as tripolar (Fig. 6D) and quadriapolar mitoses (Fig. 6E). The former
chromosome abnormalities were strikingly reduced (Fig. 6, B and
G) upon the introduction of hTERT, suggesting that the transgene was eliciting its effects on the SCC cells by correcting telomere
dysfunction. The control cell lines BICR6 and BICR31 displayed very few
dicentrics and anaphase bridges to begin with, and they expressed
even fewer when they expressed hTERT, in line with the minimal
effect of ectopic hTERT expression on the growth of these cell lines
(Fig. 5). Interestingly, control cell line BICR63, which did display a
small increase in cloning efficiency and colony size after the intro-
duction of hTERT (Fig. 5), did show a higher number of dicentric
chromosomes and anaphase bridges than BICR6 and BICR31, and
these were reduced by the expression of hTERT (Fig. 6, B and G). The
BICR63 culture may be another example of an immortal telomerase-
positive cell line that has sufficient telomerase and telomere function
to bypass M2 with suboptimal growth but not enough to counter
chromosomal instability (28, 51). Nevertheless, the above-mentioned
results suggest that BICR7 displays properties of M2 crisis *in vitro* in
part because of telomere dysfunction and not wholly due to other
possible functions of telomerase.
The BICR7 SCC Has Bypassed M0 and M1 in Vivo and Displays Properties of M2 Crisis in Vivo. Both the BICR7 culture and tumor have bypassed M0 and M1 based on their genetic profiles (Table 1), but the BICR7 culture is deficient in telomerase (Fig. 3). The next question concerned the relevance of the above observations to in vivo SCC samples. In an attempt to answer this, we quantitated the frequency of anaphase bridges in the original BICR7, BICR6, and BICR63 tumors (BICR31 tumor was not available). We found that the ABI in the BICR7 tumor (0.38) was considerably higher than that in the BICR6 tumor (0.14), with BICR63 displaying an intermediate ABI [0.29 (Figs. 6G and 7)], indicating that the original BICR7 tumor had some telomere dysfunction before being placed in vitro. Taken together, these data suggest that the BICR7 bypassed M0 and M1 and was beginning to enter a M2 crisis in vivo. The BICR7 culture increased its ABI to 0.4 by 12 MPDs and to 0.8 by 20 MPDs. At this point the BICR7 culture ceased to grow, as defined by a failure to undergo 1 MPD in 4 weeks.

From our in vitro observations (6) and the findings of others (15, 52), it appeared that squamous neoplasms in the post-M1, pre-M2 state are quite rare, and only one other possible example has been reported (15). However, it could not be excluded that most SCCs approaching M2 in vivo fail to proliferate in vitro sufficiently to establish cultures from small biopsies. To test this, we looked at 29 randomly selected OSCC samples and quantitated the frequency of anaphase bridges (Fig. 7C). Sixteen of 29 primary OSCC samples showed ABIs that were greater than that of BICR63 in vitro, the immortal SCC line with the highest ABI, and 6 of these displayed indices that were comparable to the BICR7 cells showing features of crisis in vitro. Interestingly, four tumors (14% of the total) showed no detectable anaphase bridges at all, and it is tempting to speculate that these SCCs are similar to those that have been reported to be telomerase negative in vivo (53) and to undergo replicative senescence.

Fig. 4. Ectopic expression of hTERT increases TRF length in both mortal and immortal cultures. The TRF length of genomic DNA isolated from pools of infected cells was analyzed along with the following control cell lines: irradiated and nonirradiated Swiss 3T3 feeders as controls for 3T3 contamination; and GM847 (ALT positive), HeLa (cervical adenocarcinoma cell line), and A1698 (bladder carcinoma cell line) as TRF control lines (GM847/H11005 23 kb, HeLa/H11005 6 kb, and A1698/H11005 3 kb). Note that BICR7 and 7NEO show a TRF length comparable with that of A1698 cells (3 kb), whereas the TRF length of the three immortal lines and their Neo vector-infected counterparts ranges from 4–6 kb. Introduction of hTERT increases TRF lengths in all cell lines to between 9 and 12 kb. Note that in the keratinocyte lines, there was variable levels of 3T3 feeder contamination, but the 3T3 TRF smear is discernable from those of the keratinocytes. MPDs after retroviral infection are shown along the bottom of the gel (NA designates not applicable).
This trend was highly significant by the Wilcoxon-Mann-Whitney test previously published for colon cancer progression (46). Furthermore, reduced proliferation to the metastatic state (Fig. 7) of lymph node metastases showed a reduction in ABI on their progression that was related to telomere dysfunction (see above). All six of the metastatic tumors did appear to show a small but significantly compromised growth rate compared with the 7NEO control culture. In contrast, there was no significant difference in cloning efficiency in BICR31 or the other two immortal cell lines, BICR6 and BICR63, when hTERT and NEO plates were compared. However, it is worth noting that 63 TERT cultures did consistently show an increase in colony size (data not shown). TUNEL assays were performed to estimate apoptosis levels in BICR7 and BICR31 cell lines infected with pBabeneo or pBabest. A 3-fold decrease in TUNEL-positive cells was observed in 7TERT cultures.

**Fig. 5.** M2 crisis displayed by BICR7 can be overcome by ectopic expression of hTERT. A, colony forming assays were carried out to compare the growth properties of BICR7 and the immortal cell lines infected with pBabeneo or pBabest. Photographs of BICR7 and BICR31 NEO- and TERT-infected plates stained with rhodamine B are shown. Ectopic expression of hTERT increased the cloning efficiency in BICR7 cultures (7TERT) approximately 8-fold compared with the 7NEO control culture. In contrast, there was no significant difference in cloning efficiency in BICR31 or the other two immortal cell lines, BICR6 and BICR63, when hTERT and NEO plates were compared. However, it is worth noting that 63 TERT cultures did consistently show an increase in colony size (data not shown). B, TUNEL assays were performed to estimate apoptosis levels in BICR7 and BICR31 cell lines infected with pBabeneo or pBabest. A 3-fold decrease in TUNEL-positive cells was observed in 7TERT cultures.

**in vitro** (6, 7). On the other hand, this subset of tumors may have very high levels of telomerase that have totally restored telomere function. No anaphase bridges were seen in any of the adjacent normal oral mucosa included with the tumor samples. Therefore, these results show that more tumors with the properties of BICR7 exist *in vivo* than is indicated by cell culture studies, and the results with the BICR7 cultures suggest that such tumors should experience a growth disadvantage as a result of M2 crisis.

**ABI Intratumor Heterogeneity.** To test whether the differences in ABI between different tumors could be explained on the grounds of intratumor heterogeneity, we measured the ABI in three different areas of two large SCCs. The first tumor had ABIs of 0.125, 0.29, and 0.33 in the three areas analyzed (average ABI, 0.29), and the second had ABIs of 0.2, 0.3, and 0.5 (average ABI, 0.3). Therefore, these data could not fully explain the differences between tumors. Furthermore, with the possible exception of 2 of the 29 tumors analyzed, there was no evidence for a clustering of the anaphases with bridges.

**OSCC Keratinocytes with Telomere Dysfunction Have a Selective Disadvantage During Tumor Progression in Vivo.** To test whether telomere dysfunction was tolerated by SCCs throughout tumor progression *in vivo*, we examined the ABI of six lymph node metastases derived from five of the randomly selected OSCCs with ABIs higher than that of BICR63 *in vitro* (0.2). The ABI found in the BICR63 culture was chosen as the cutoff point because this cell line did appear to show a small but significantly compromised growth rate that was related to telomere dysfunction (see above). All six of the lymph node metastases showed a reduction in ABI on their progression to the metastatic stage (Fig. 7D), in line with the overall trend published previously for colon cancer progression (46). Furthermore, this trend was highly significant by the Wilcoxon-Mann-Whitney test (*P* < 0.03). In contrast, seven primary tumors that had low ABIs to begin with did not show such a trend upon metastasis to the lymph node (*P* > 0.5). One primary tumor with an ABI of 0.15 actually showed nearly a 2-fold increase on progression from the primary to the metastatic state. This anomaly may be a rare example of a case where senescence breaks down late in the progression of SCC so that the ABI is still rising as the tumor approaches crisis. We have previously reported that one primary tumor BICR21 evolved the immortal phenotype only at the metastatic stage (BICR22; Ref. 6) and that this coincided with the clonal expansion of the p53 mutation found in BICR22 but barely detectable in BICR21 (54). These results strongly suggest that in OSCCs, levels of telomere dysfunction above a certain threshold are not well tolerated and are eventually selected against during tumor progression *in vivo*.

**DISCUSSION**

In this study, we provide direct evidence that human cancer cells can exist in an interim state of telomerase deficiency, even when they have bypassed the M0 and M1 senescence checkpoints. Our data also showed that not all tumor cells that express detectable telomerase activity by the TRAP assay are actually immortal, thus offering a possible explanation for the discrepancy between the fraction of SCCs that are immortal *in vitro* (60%; Ref. 6) and telomerase positive *in vivo* (90%; Ref. 53).

Our observations support the notion that at least some human SCCs arise from telomerase-deficient cells, following the sequential inactivation of M0 and M1, rather than from preexisting telomerase-competent cells (39). These tumors most likely reactivate or up-regulate telomerase to bypass M2 (10–12) because ALT has never been reported in this tumor type, and the ectopic expression of telomerase enabled cultures of the pre-M2 tumor BICR7 to bypass M2 and become immortal *in vitro*. In tumors such as BICR7, the malignant cells may arise from the more abundant, differentiated parts of the tumor, which are telomerase negative (34–38). Alternatively, the levels of functional telomerase in the normal basal cells of squamous epithelium may be too low to sustain functional telomeres, rendering the tumor telomerase deficient from the point of initiation, and the latter hypothesis is consistent with the reported reduction in skin average TRF lengths with increased donor age *in vivo* (55).
It is rare to find in vitro SCCs that have the post-M1, pre-M2 characteristics of BICR7, and only one possible previous example, that of an oral dysplasia, has been reported (15). One trivial possibility might be that the culture conditions used by most investigators are suboptimal (17). However, we have used the best available methods for keratinocyte culture (15, 17), and we exhaustively tested the culture requirements of each SCC we placed into culture (see Ref. 6 and “Materials and Methods”). Another possibility is that M2 crisis occurs earlier in SCC progression than the carcinoma stage, but a survey of 21 oral dysplasia cultures by our colleagues (52) and ourselves (7) did not reveal any cultures with the properties of BICR7. However, ABIs of 29 primary OSCCs indicated that the frequency of tumours approaching M2 in vivo might be rather higher than that revealed by culture studies (20% versus 5%), probably because tumours such as BICR7 do not proliferate well enough to establish cultures in vitro.

Our data show that the telomerase-deficient BICR7 tumor population is severely compromised in its ability to grow in vitro and that if this situation were repeated in comparable in vivo tumors (Fig. 7D), a considerable selection pressure in favor of tumor cells with increased telomerase and/or telomere function would be predicted. Our study and recent observations made in a study of human colon cancer progression (46) are in accord with this suggestion because it was noted that the ABI fell when the tumors progressed from the primary to the metastatic site, suggestive of passing M2 crisis. Immortal human cell lines can show some evidence of telomere dysfunction without their growth rates being greatly compromised (Refs. 28 and 51; Figs. 3 and 5), and indeed previous work on in vitro model systems showed that human cells can tolerate a low level of telomere dysfunction when they pass through M2 (12). However, it should be noted that in the above in vitro studies, nearly all of the cultures with the highest ABIs were not immortal (12, 28, 46, 51).

A high level of telomere dysfunction may aid the ability of human cells to bypass M2, and a low level of telomere dysfunction may be beneficial for tumor progression once M2 is bypassed. It has been suggested that human cancers in vivo may be able to tolerate anaphase bridges (13, 28) and that this may generate certain types of chromosome rearrangement, especially when p53 function is compromised (56). However, our data and that of others (46) suggest that human tumors with the highest ABIs suffer a growth disadvantage until telomere dysfunction is reduced to a tolerable level. It should be stressed that dicentric chromosomes and anaphase bridges can be generated by mechanisms other than low levels of telomerase (57, 58).

These include the dysfunction of numerous telomere-associated...
and DNA repair proteins (57) and the overexpression of the proto-oncogene c-myc (58). However, in all four SCC lines that we studied, the ABI was repressed by the ectopic expression of telomerase. This is not consistent with any of the above alternative mechanisms except for deficiencies in the Blooms and Werner’s helicases and in Ataxia Telangiectasia Mutated. There is no evidence for alterations of these proteins in human SCC, and furthermore, previous studies using our SCC lines have failed to note any increase in c-myc expression (59, 60). Therefore, we favor insufficient levels of telomerase as the most likely explanation for the presence of high ABIs in our SCC samples.

The overexpression of telomerase corrected both telomere dysfunction and poor growth rate in our study, and this would be consistent with the gradual increase in telomerase levels observed during the progression of many advanced human tumors, including OSCC (53, 61). Our data cannot yet exclude the possibility that some SCCs arise from telomerase-competent keratinocytes, but we think most bypass M0, M1, and M2 sequentially, for the following reasons.

Mice engineered to be deficient for telomerase and haploinsufficient for p53 develop carcinomas harboring imbalanced chromosomal translocations typical of their human counterparts (56), suggesting that a combination of dysfunctional telomeres and p53 creates the necessary genomic instability for human epithelial cells to evade M2 crisis. Other data support this conclusion; virtually all immortal human SCC lines have p53 dysfunction (6, 24) and show imbalanced translocations plus many chromosomal gains and losses, including Robertsonian translocations. The latter are symptomatic of telomere dysfunction earlier on in the evolution of the tumor (56), and interestingly, one of the immortal control lines used in our study, BICR6, has a Robertsonian translocation of chromosome 14 (data not shown). Moreover, carcinomas that are still capable of replicative senescence have wild-type p53 genes (6) and possess stable genomes because very few chromosomal gains and losses are detectable in these cultures by comparative genomic hybridization (62), and there is a low frequency of loss of heterozygosity (6). Taken together, these data, when combined with those of our current study, suggest that the clonal proliferation of SCC cells in a telomerase-deficient setting creates the background for chromosomal instability and the subsequent bypass of M2 crisis (46, 56), probably by up-regulating telomerase.

Our data also show that, at least in OSCCs, a significant fraction of the tumors show substantive telomere dysfunction. We also show that telomere dysfunction is due to inadequate telomerase and that in contrast to other reports (28), it can severely compromise tumor growth by precipitating mitotic failure and cell death. Assuming that our interpretation is correct, it is likely that the subset of SCCs with
high ABIs is the subset with the shortest TRF lengths (63). Moreover, because these tumors are already displaying telomere dysfunction in the absence of any intervention, our results support the contention that the growth of this type of tumor in vivo would be inhibited after therapeutic inhibition of telomerase (29, 30), without necessarily displaying an unacceptable lag phase.

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High Levels of Telomere Dysfunction Bestow a Selective Disadvantage During the Progression of Human Oral Squamous Cell Carcinoma

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