Lysis of Dysplastic but not Normal Oral Keratinocytes and Tissue-Engineered Epithelia with Conditionally Replicating Adenoviruses

Kamis Gaballah, Allison Hills, David Curiel, Gunnel Halldén, Paul Harrison, and Max Partridge

1Head and Neck Cancer Unit, King's College London, Guy's, King's and St. Thomas' Hospitals, and 2Centre for Molecular Oncology, Institute of Cancer and the Cancer Research United Kingdom Clinical Centre, Barts and The London, Queen Mary’s School of Medicine and Dentistry, London, United Kingdom; 3Division of Human Gene Therapy, University of Alabama at Birmingham, Birmingham, Alabama; and 4Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow, United Kingdom

Abstract

There is no effective medical treatment for oral precancer, and surgery to remove these lesions is imprecise because abnormal mucosa extends beyond the visible lesion. Development of vectors for tumor-selective viral replication has been a significant advance, and viral lysis is well suited to destruction of oral precancerous mucosa. To facilitate evaluation of new treatments, we engineered dysplastic oral epithelium using keratinocytes isolated from dysplastic lesions. We show that these model systems recapitulate the key characteristics of the clinical lesions closely, and that topical delivery of the conditionally replicating adenovirus (CRAd) d922-947 can lyse tissue-engineered epithelia that show mild, moderate, or severe dysplasia, but normal oral epithelia are very resistant to this treatment. The lytic effect is determined by various factors, including the grade and proliferation index of the dysplastic epithelia. The presence of suprabasal cycling cells, expression of the coxsackie adenovirus receptor (CAR), the transcription cofactor p300, and other aberrations that affect the regulation of the cell cycle or apoptosis and promote viral replication may also be important. The ability of d922-947 to destroy engineered oral dysplasia was significantly greater than that observed using wild-type adenovirus, d/1520, or viruses modified to bypass cell entry dependent on the presence of CAR. Evidence of infection in clinical dysplastic lesions was also shown ex vivo using tissue explants. We conclude that d922-947 may provide an efficient molecular cytotoxic to dissolve oral dysplastic lesions. [Cancer Res 2007;67(15):7284–94]

Introduction

The results following treatment for oral cancer are disappointing because only half of all cases survive for 5 years after diagnosis. Locoregional recurrence is the most common cause of treatment failure, but a second tumor may also develop in a patch of precancerous mucosa. Surgery to remove these abnormal patches is unreliable because whereas some precursor lesions produce visible thickening or thinning of the mucosa, genetic aberrations extend well beyond the visible lesion, and there is no way of precisely defining where the margins of the lesions lie (1–5). There is no medical treatment that can effectively eliminate these precancerous patches, and innovative approaches that can prevent progression to malignancy are required.

Selective replication of adenoviruses offer the potential to destroy precancerous lesions because they replicate in dividing cells. They are particularly useful for destroying lesions at accessible sites because they can be delivered topically and treatment can be repeated on multiple occasions. Several classes of conditionally replicating adenovirus (CRAd) have been developed to restrict viral replication to tumor cells by introducing modifications that abrogate viral functions that are essential for replication in normal cells but redundant in tumors. d922-947 (6) and D24 (7) are examples of pRb-binding deficient CRAds with abrogated viral function based on deletions in E1A proteins. These abolish the binding of E1A to pRb family proteins, preventing release of the E2F transcription factor, activation of the adenovirus E2 promoter and cell cycle regulatory proteins that allow S-phase entry and replication in quiescent cells. These viruses can theoretically only replicate in cells where E2F is deregulated.

The coxsackie adenovirus receptor (CAR) is the primary receptor for adenoviruses, but internalization requires αv integrins. Because tumor cells frequently show reduced expression of these receptors (8–11), a second class of CRAds have been modified to bind to alternative receptors. D24RGD incorporates an Arg-Gly-Asp (RGD) sequence in the fiber that interacts with CAR and αv integrin (12), and Ad5/3Δ24 (13) uses the Ad3 receptors CD46 (14), CD80 (B7.1), and CD 86 (B7.2; ref. 15) for internalization.

The most extensively studied CRAd is d/1520 (ONYX 015) designed to selectively replicate and destroy p53-mutant cells (16). This virus carries an inactivating deletion encoding the E1B-55kD protein that inactivates p53 during viral replication, inhibiting p53-mediated apoptosis. Several tumor cell lines with intact p53 support replication of d/1520 and carry defects in p53-dependent pathways (17, 18). However, the oncolytic properties of this virus are attenuated due to the presence of the E1B deletion. In a pioneering report, Rudin et al. (19) showed that daily application of d/1520 as a mouthrinse could reduce the severity of oral dysplastic lesions, providing initial evidence that CRAds can penetrate dysplastic epithelia. Efficient replication of CRAds and evidence of viral lysis have been observed maintaining tumor explants ex vivo (20). However, normal tissues and dysplastic lesions may rapidly undergo autolysis, and we were unable to conclusively show viral replication using tissue explants. Recognizing these limitations, we developed a new three-dimensional culture system maintaining oral keratinocytes derived from dysplastic lesions on fibroblast-containing collagen gels. The...
phenotypic characteristics of the engineered dysplastic and oral epithelia were compared with normal and lesional oral mucosa to show that they recapitulate the tissue morphology closely. These model systems were used to study the response of oral keratinocytes to wild-type adenoviruses, dl922-947, Δ24, $d_{1/520}$, Δ24RGD, and Ad5/Δ24. We show that the ability of dl922-947 to dissolve dysplastic and malignant oral epithelia was superior to the other modified viruses, and that this treatment had minimal toxicity in normal oral epithelia.

Materials and Methods

Tissues. The study was approved by the Ethics Committee of King’s College Hospital. Informed consent was obtained from all patients. Samples of normal oral mucosa were obtained from non-, para-, and fully keratinized sites. Lesional mucosa was obtained from portions of biopsy material that were not selected by the pathologist for light microscopy.

Monolayer culture. Normal oral keratinocytes (NOK) were grown from tissue explants and maintained with a J2 fibroblast feeder layer (21). Established cultures of oral keratinocytes derived from lesions showing moderate/severe dysplasia D6 (22), mild/moderate dysplasia DOK (23), moderate dysplasia D20 (22), and moderate/severe dysplasia POE9n (24) were also grown with a J2 feeder layer. Four new strains of dysplastic oral keratinocytes were evaluated. LDK was established from a severe dysplasia on the lingual alveola. This strain carries a p53 gene mutation (G7-7 at codon 248) and does not express p16. CDOK was established from a mild dysplasia at the commissure. LTDOK was established from a mild dysplasia on the lateral tongue, and SPDK from a moderate dysplasia on the soft palate. These strains and D6 are all mortal, wild type for p53, and express p16. D20, DOK, and POE9n carry a p53 gene mutation and do not express p16 (21–23); D20, DOK, and LDK are immortal, and POE9n has an extended culture life span. The JHU022 (8), HN5 (25), and CAL 27 [CRL-2094, American Type Culture Collection (ATCC)] squamous carcinoma cell lines were maintained in DMEM, Hams-F12, and 10% fetal bovine serum (Invitrogen).

Preparation of engineered oral epithelia. Keratinocytes (1 × 10⁶) derived from normal oral epithelium (p2) and dysplastic lesions (p2-18) were grown on collagen gels and raised to the air-medium interface on day 2 (26). In some experiments, keratinocyte growth factor (KGF; Invitrogen) was substituted for epidermal growth factor (EGF). The grade of dysplasia was established for each epithelium by considering the changes in architecture and cytologic features.

Recombinant adenoviruses. We evaluated dl922-947, Δ24, d1/520, Δ24RGD, and Ad5/Δ324, Δ24 and dl922-947 are similar viruses with deletions at 923 to 946 and 922 to 947 bp, respectively. Ad5GFP is E1 deficient and carries the gene for green fluorescent protein (GFP) under control of the cytomegalovirus promoter; dl312 has a total E1A gene deletion. These viruses and Adwt were propagated on 293 cells and purified using cesium chloride density gradient centrifugation.

Cytotoxicity assays. The cytotoxicity of the viruses was established by MTS assay. Keratinocytes were plated at 1 × 10⁶ with a J2 feeder layer and after 24 h were either mock infected or infected with a range of viral particles (vp) for 90 min in serum-free media (SFM). After 7 days, MTS assay (Promega) was added, and the absorbance was measured at 490 nm. The keratinocytes were also grown to 40% confluence before being infected with 10 vp per cell. Lysates were resolved by PAGE, and membranes were transferred to nitrocellulose. Cell cycle analysis was done 48 h following infection of the keratinocytes.

Viral infection of tissue explants. Explants were prepared from clinical dysplastic lesions ($n = 10$) and normal oral mucosa ($n = 10$) and exposed to 10⁵ vp of dl922-947, Adwt or d312 in SFM, adding the virus to the uppermost layer of the culture for 2 h. After 7 to 14 days, each culture was divided into two. One-half was snap frozen in liquid nitrogen; the other was fixed in formalin. Cytopathic effect and general tissue morphology was assessed. The E1A receptor was detected with GRO1L (1:40; Oncogene Science, DAKO), E cadherin with BTA1 (1:200; R&D Systems), CD44 v6 with VVF-7 and v7 with VVF-9 (1:50–1:200; Abcam), occlnin with the polyclonal antibody (1:100; Zymed, Invitrogen), laminin 5 with D4BS (1:200; Chemicon Chancellors Ford), collagen 4 with C1V22 (1:50; DAKO), fibronectin with IST-9 (1:200; Abcam), p16 with F-12 (1:50; Santa Cruz, Autogen Bioclair), ov3 with clone 1976 and ov3 with clone 1976 (1:50; Chemicon), and filagrin with BT-576 (Biogenesis). Immunolocalization of cytokeratins 10, 13, and 19 was with 9205, BA16, and 1384-500, respectively (all 1: in 50; all Abcam). p300 was detected with MS-586-XMI (1:20; Lab Vision) and Ki67 with MT240 (1:50; DAKO). Sections stained with monoclonal antibodies were incubated with biotin-conjugated rabbit anti-mouse antibody followed by streptavidin-horseradish peroxide complex (Dako). The KI67 proliferation index was determined for the basal and suprabasal compartments of the epithelium as the mean percentage of positive cells ± SE in five contiguous fields at 100× magnification. Immunoreactive cells were counted using Qwin image analysis software. A similar protocol was followed when assessing the proliferation index for monolayers, analyzing five fields captured at random at 100×.

Viral early gene expression was detected with anti-E1A (1:50; Oncogene Science) and quantitated by counting the number of cells that were strongly positive as E1A “spots” for five fields at 100×. Viral hexon protein was detected with anti-Ad5 antiserum (1:200; Cocalico Biologicals).

Fluorescence-activated cell sorting analysis and Western blotting. Cell cycle analysis was done 48 h following infection of the keratinocytes with 10 vp per cell. Lysates were resolved by PAGE, and membranes were probed with antibodies for p16, Rb, RbP, cyclin D1, cyclin E, Ecd4, and Ekd6 (1:1,000, clones 554079, 554136, 9308, 554180, 559693, 554086, 554182; all BD PharMingen, Abcam).

Statistical analysis. Statistical analyses were done using SPSS. A descriptive analysis was done initially followed by one-way ANOVA. Comparison of the lysis scores was with StatXact3 for Windows using Xact nonparametric inference to allow for small sample size.

Results

Characterization of the G₁-S checkpoint for dysplastic oral keratinocytes. We investigated the expression of key cell cycle proteins for the normal, dysplastic, and malignant keratinocytes. We found an increase in the levels of RbP in the immortal dysplastic keratinocytes, the JHU022 cell line and for the POE9n strain (Fig. 1A). Higher levels of hypophosphorylated Rb were present in D6 and NOK. p16 was expressed by all mortal dysplastic keratinocytes, but was absent from POE9n, D20, DOK, and LDK. Immunohistology confirmed the expression of p16 by D6, SPDK, CDOK, and LTDOK. The immortal dysplastic strains showed increased expression of cyclin D1, but levels of the cdks were consistent for all cell types.

Lysis of dysplastic and malignant but not NOKs after infection with dl922-947 or Δ24 in monolayer. Oral keratinocytes derived from normal and dysplastic lesions were infected with dl922-947, Δ24, Adwt, and d1/520 to assess the cytopathic effect (Fig. 1B). The MTS assay showed that lysis was most effective for the malignant and dysplastic keratinocytes, with the
percentage of viable cells after infection with 10 vp per cell of 
\(d922-947\) in the range 20% (JHU022) to 68% (POE9n), with an 
average of 43% for the five strains of dysplastic keratinocytes 
onlysis, with an average of 43% for the five strains of dysplastic keratinocytes 
on infection with 10 vp per cell and 87% viable cells remained, but they were readily lysed 
by Adwt (42% viable cells, \(P < 0.005\)). The efficiency of the lytic response was compared for the D6, POE9n, and D20 dysplastic oral keratinocytes, selected as examples of strains with a finite, 
extended, and immortal phenotype, by estimating the amount of 
\(d922-947\) required to produce 40% lysis. This comparison showed that 
the D6 strain lysed most readily followed by D20, but that the 
POE9n stain did not reach this threshold even after exposure to 
1,000 vp per cell.

The D20, DOK, and POE9n strains, together with NOK, were also 
exposed to \(d/1520\). This CRAd produced only limited lysis in comparison to the effect of \(d922-947\), and after exposure to 10,000 
vp per cell, 88%, 72%, and 96% viable cells remained, respectively, 
but NOK were very resistant to lysis, with \(d922-947\) at 10 vp 
per cell and 87% viable cells remained, but they were readily lysed 
by Adwt (42% viable cells, \(P < 0.005\)). The efficiency of the lytic response was compared for the D6, POE9n, and D20 dysplastic oral keratinocytes, selected as examples of strains with a finite, 
extended, and immortal phenotype, by estimating the amount of 
\(d922-947\) required to produce 40% lysis. This comparison showed that 
the D6 strain lysed most readily followed by D20, but that the 
POE9n stain did not reach this threshold even after exposure to 
1,000 vp per cell.

**Infection with \(d922-947\) or \(\Delta 24\) induces S phase in 
dysplastic but not NOKs.** Infection of the D6, POE9n, and D20 
strains with 10 vp of \(d922-947\) induced a 3.3-, 2-, and 4-fold increase, respectively, in the number of cells in S phase when 
compared with the E1-deleted CRAd, \(d/312\) \((P < 0.05)\, but the 
number of cycling normal keratinocytes was not changed 
significantly. As anticipated, Adwt induced a significant increase 
in the number of cycling cells for all strains (Fig. 1C). Induction of 
S phase in dysplastic oral keratinocytes was confirmed by an 
increase in the proliferation index following infection with 10 vp 
per cell of \(d922-947\) or Adwt for all strains. Representative examples 
are shown for CDOK and NOK (Fig. 1D), together with the pattern 
of expression of Ki67 and E1A.

**Infectivity of normal and dysplastic oral epithelium ex vivo.** 
Explants of normal and dysplastic oral epithelium showed varying 
degrees of autolysis within a few hours, and some had disintegrated 
by 24 h, although the basal keratinocytes were often retained. 
Expression of hexon and E1A was detected in some cells at the 
edges of 4 of 10 explants of dysplastic tissue 30 h after infection 
with \(d922-947\), or Adwt (Fig. 2A), but extensive autolysis precluded 
further experimentation.

**Morphologic and phenotypic characterization of tissue-
engineered oral epithelia.** Morphologic assessment of the 
epithelia generated in vitro using keratinocytes derived from 
nonkeratinized sites showed a multilayered epithelium resembling 
the parent tissue with a polarized basal layer and larger spinous 
cells (Fig. 2B). For keratinized epithelia, the upper cells became 
flattened as keratohyaline granules accumulated. Rete ridges were 
rarely observed, but normal differentiation was confirmed by 
suprabasal expression of keratin 13 for nonkeratinized oral 
epithelia (>60% positive cells) and keratin 10 (20–40% positive 
cells) for keratinized epithelia. The patterns of expression of the 
EGF receptor, CD44, E cadherin, and occludin were as obtained for 
normal oral mucosa. The rate of proliferation of the nonkeratinized 
oral epithelia was greater than for keratinized tissue, mimicking the 
pattern observed in vivo (28).

Maintaining the dysplastic keratinocytes with EGF, as opposed 
to KGF, supported the development of epithelia where the severity 
of the dysplasia was similar to that reported for the parent lesion. 
These epithelia showed architectural and cytologic disturbances 
that were scored as either mild (DOK, SPDOK), moderate (D6, 
CDOK), moderate to severe (POE9n, D20) mirroring the changes 
found in the in vivo dysplasias (Fig. 2A). Dysplastic keratinocytes 
isolated from two lesions yielded tissue-engineered epithelia with a 
phenotype that was graded as more severe than the parent tissue 
(LTDOK-clinical lesion moderate dysplasia, engineered epithelia 
severe dysplasia; LTDOK-clinical lesion severe dysplasia, engineered 
epithelia carcinoma in situ). The basal layer was frequently not 
polarized, and suprabasal mitoses were observed in the dysplastic 
epithelia generated with the D6, POE9n, D20, and LTDOK strains. 
The Ki67 proliferation index for the epithelia generated with mortal 
dysplastic strains (p2–4) was lower than anticipated when 
compared with NOK, but epithelia derived using the immortal 
dysplastic strains showed higher rates of proliferation and frequent 
suprabasal mitoses, mimicking the in vivo scenario.

The pattern of keratinization was variable with dysplastic 
keratinocytes with a finite life span generating thin, highly kerati-
nized epithelia, whereas the immortal strains produced cultures that 
were thicker and less well keratinized. Cytokeratin 13 was expressed 
in the suprabasal layers of the tissue-engineered epithelia prepared 
with D6, CDOK, LTDOK, and SPDOK (40–60% positive cells), DOK 
(>60% positive), D20 and POE9n (20–40% positive), LTDOK (>20% 
positive) and in the basal layer for JHU022. Cytokeratin 10 was 
expressed by the DOK and D20 epithelia (20–40% positive). No drop-
shaped rete pegs were observed, and the engineered epithelia lacked 
the hyperplasia and excessive surface keratinization that may be 
a feature of clinical lesions. The engineered dysplastic epithelia 
expressed the EGF receptor and E cadherin on more cell layers than 
found for the matched normal tissue, mimicking the findings for 
in vivo dysplastic lesions. Further detailed characterization of ex-
pression of keratin 19, filaggrin, fibronectin, CD44, occludin, collagen 
4, and laminin 5 showed patterns characteristic for a range of clinical 
lesions showing mild (n = 12), moderate (n = 8), or severe (n = 6) 
dysplasia (data not shown). The v\(\alpha3\) and v\(\alpha4\) integrins were 
detected in the basal and lower spinous cells of normal oral mucosa 
and dysplastic lesions, but the engineered dysplastic epithelia 
showed only low (D20 and DOK) or no expression (POE9n) of these 
subunits. However, these integrins were visualized at the cell 
membranes for the engineered D6 and DOK epithelia. The staining 
also seemed to be intracellular and to diffuse into the collagen gel. 
Comparison of the immunophenotypic profile for the DOEs with the 
matched clinical lesion (CDOK, SPDOK, LTDOK) showed increased 
expression of keratin 13 for the engineered tissue equivalents (on 
average, 15% more positive cells) and a reduced Ki67 index (data not 
shown) when compared with the matched clinical lesion, but all 
other markers were similar. These phenotypic changes were 
consistent for epithelia generated with the immortal keratinocytes 
maintained through serial passage. The JHU022 cell line generated 
an epithelium that resembled noninvasive carcinoma in situ (data 
not shown).

**Infection of tissue-engineered oral epithelia with AdGFP.** 
When the engineered normal oral epithelia were infected with 
AdGFP, expression of GFP was found only after infection with 
100 vp per cell and limited to the uppermost cell layers (Fig. 2B). 
This indicates that the virus is able to gain entry into the super-
ficial cells due to disruption of tight junctions as each differenti-
tiating layer is lost, but it is unable infect the deeper layers. When
the dysplastic counterparts were infected with this virus, the pattern was as described for the normal epithelia, although the LDK strain expressed GFP in the spinous layer at sites where loss of cohesion was apparent (Fig. 2B).

**Figure 1.** Characterization of dysplastic oral keratinocytes and demonstration of the lytic effect of CRAds for oral dysplasia in monolayer culture. A, levels of RbP, p16, and cyclin D1 in normal (NOK), dysplastic (D6, SPDOK, CDOK, POE9n, DOK, D20, LDKOK), and malignant (JHU022) oral keratinocytes. The expression of the proteins was measured by Western blotting using mitogen-activated protein kinase as a loading control. B, cell death induced by Adwt, dl922-947, d/1520, Δ24RGD, and Ad5/3Δ24 in normal and dysplastic oral keratinocytes after infection with 10 vp per cell and 1,000 vp per cell. The D6 strain showed most lysis following infection with dl922-947, and Ad5/3Δ24 is more lytic than Δ24RGD at 10 vp per cell. NOKs showed more lysis that the D20 dysplastic strain when exposed to d/1520 at 1,000 vp per cell. C, induction of S phase following infection of NOK, D6, POE9n, and D20 with 10 vp per cell of d312, dl922-947, d/1250, or Adwt (see key below). Exposure to dl922-947 and Adwt increased the proportion of dysplastic oral keratinocytes in S-phase, but the normal cells only showed S phase induction after exposure to Adwt. D, the increased Ki67 proliferative index for NOKs and the CDOK dysplastic strain following exposure to Adwt and for the CDOK strain after exposure to dl922-947. The pattern of positive Ki67 and E1A staining following exposure of the CDOK strain to dl922-947 confirmed that this CRAAd induced S-phase in dysplastic but not normal keratinocytes.

**Tissue-engineered normal oral epithelia are very resistant to lysis by dl922-947 or Δ24.** Infection of tissue-engineered normal oral epithelia derived from 9 of 12 strains of oral keratinocytes from nonkeratinized sites with dl922-947 or Δ24...
produced an increase in keratinization but no evidence of lysis. Two strains showed very limited lysis and a single stain of buccal keratinocytes foci of ballooning degeneration with loss of cells from the tissue, but the surface layers were retained intact. Similarly, very limited lysis was observed for 2 of 19 samples of engineered epithelia derived from para- or fully keratinized normal oral mucosa following exposure to \( dl_{922-947} \) or \( \Delta 24 \). In contrast, infection with Adwt induced more lysis in all engineered normal epithelia (Fig. 2A).

**Efficient lysis of tissue-engineered oral dysplastic epithelia following infection with \( dl_{922-947} \) or \( \Delta 24 \).** The lytic effect of \( dl_{922-947} \) and \( \Delta 24 \) was dose dependent for all engineered oral dysplasias evaluated, and the lysis score was significantly higher when epithelia prepared with the dysplastic and normal strains were compared following exposure to 100 vp per cell \((P = 0.0222, \text{Table 1})\). A reverse effect was found after infection with Adwt \((P = 0.0222)\). Cytopathic changes included an increase in the level of superficial keratinization, the presence of bursting cells, ghost cells, and superficial debris. These changes seemed more rapid in the spinous and basal layers as the number of vp was increased. Examples of the patterns of lysis are shown for the D20, DOK, POE9n, and D6 strains (Fig. 3A). Efficient dissolution of the D20 and DOK epithelia after exposure to 100 vp per cell is shown at days 7 and 12, respectively. As the lytic foci coalesced, cavities filled with fluid and floating cells were often detected, and gross disturbances in the tissue architecture and marked cytologic changes were apparent as the tissue dissolved. The pattern of lysis of the POE9n and D6 epithelia, after exposure to 100 vp per cell, is also shown (Fig. 3A). The LDK epithelia also showed very efficient lysis with complete loss of all dysplastic keratinocytes by day 7. Infection of these epithelia with Adwt produced a more limited response (Table 1), although occasional foci of more extensive lysis were observed (Fig. 2, bottom, right).

The pattern of expression of the viral hexon protein showed that \( dl_{922-947} \) or \( \Delta 24 \) reached the deeper layers of the dysplastic epithelia after exposure to 10 vp per cell for the D20 and LDK strains that lysed most efficiently (Fig. 3B). Expression of hexon protein also increased as viral replication and lysis occurred. Dysplastic epithelia with a lower lysis score, for example, that derived from the POE9n strain, showed a more heterogeneous pattern of staining.

**Evidence of replication of \( dl_{922-947} \) or \( \Delta 24 \) in tissue-engineered oral epithelia.** Evidence of viral infection after exposure to \( dl_{922-947} \) was confirmed by expression of E1A with the highest number of E1A spots counted for the malignant and immortal dysplastic epithelia (Figs. 3C and 4A). The mortal strains showed expression of E1A despite having a low Ki67 proliferative index. Evidence of replication of \( dl_{922-947} \) and Adwt was shown for all infected dysplastic epithelia with the highest number of infectious units detected for the strains showing the most lysis (Fig. 4B).

---

**Figure 2.** Dysplastic oral epithelia infected with \( dl_{922-947} \), summary of the phenotypic similarities of dysplastic oral lesions and tissue-engineered dysplastic oral epithelia, morphologic characteristics of representative engineered normal and dysplastic epithelia and the lytic effect of Adwt. A, oral dysplastic tissue infected with \( dl_{922-947} \) to determine the infectivity of clinical lesions by staining for intranuclearexpression of E1A as an indication of viral replication 30 h postinfection in culture, together with the mock-infected control at low and high power (LP, HP). These tissues also show evidence of autolysis. B, H&E-stained sections of normal (NOK) and representative examples of tissue-engineered dysplastic oral epithelia, including those prepared from the D20 strain maintained with KGF (mild dysplasia), D20 maintained with EGF (severe dysplasia), DOK (mild dysplasia), and LDK (carcinoma in situ). C, transduction with AdGFP showed that expression of this marker protein was limited to the superficial layer for engineered normal oral epithelia and to focal areas of the upper and middle layers of the engineered LDK dysplastic epithelia. The pattern of expression of E cadherin is also shown for this epithelium. The lytic effect of Adwt was greater for tissue-engineered oral epithelia prepared using NOK after exposure to this virus at 100 vp per cell for 14 d when compared with the engineered dysplastic oral epithelia, although occasional foci of more extensive lysis were detected as typified by the response of the LDK strain.
The POE9n epithelia and 4 of 16 clinical dysplastic lesions showed cell coralepitheliamodulatetheseverityoflysiswith areas where the superficial cells expressed this receptor, (Fig. 5 for CAR on the basal and middle cell layers, and there were many keratinized sites. Most engineered dysplastic oral epithelia receptor was reduced on the upper differentiating cells at the epithelium at nonkeratinized sites, and that expression of this revealed that CAR was detected at cell membranes throughout lyzed completely (Table 1).

engineered oral epithelia, with a low Ki67 proliferative index, also frequent suprabasal mitoses, was infected with basal cells remained when the POE9n epithelia, which shows is not the only factor influencing the extent of lysis because some dl suprabasal mitoses were detected (Table 1), and after exposure to epithelia was maintained with EGF, as opposed to KGF, more the most efficient lysis after exposure to dl

Our detailed survey of patient-derived normal oral mucosa revealed that CAR was detected at cell membranes throughout the epithelium at nonkeratinized sites, and that expression of this receptor was reduced on the upper differentiating cells at keratinized sites. Most engineered dysplastic oral epithelia showed strong membranous and or weak cytoplasmic staining for CAR on the basal and middle cell layers, and there were many areas where the superficial cells expressed this receptor, (Fig. 5B). The POE9n epithelia and 4 of 16 clinical dysplastic lesions showed areas where there was almost no CAR on the basal cells, although immunoreactive cells were found at higher levels and for other patches of basal cells at different sites along the basement membrane (Fig. 5B). The D20 and DOK epithelia showed only low levels of expression of ov integrin, yet supported lytic viral replication and lysis.

We examined the pattern of expression of p300, a transcription factor that can initiate DNA synthesis and facilitate viral and cellular DNA replication (24) because these viruses retain the sequences that bind to this cofactor. We found that p300 was restricted to the basal cells of nonkeratinized tissues but detected throughout the epithelia in vivo and in vitro at keratinized sites (Fig. 5C). The mortal engineered dysplasias expressed this cofactor throughout the tissue (Fig. 5C), whereas those generated with the immortal stains, and the clinical dysplastic (Fig. 5C) and malignant lesions, showed a more variable pattern, with some cultures and tissues showing strong staining for this cofactor, whereas others had a more focal pattern.

Δ24RGD and Ad5/3Δ24 modified to enter epithelial cells via different receptors do not lyse tissue-engineered oral dysplasia efficiently. The cytotoxicity of Δ24RGD and Ad5/3Δ24 for normal and most dysplastic oral keratinocytes, maintained as monolayers, was less than for dl922-947. The D20 strain was most sensitive to these viruses, but the 40% cell-killing threshold was only reached following infection with 1,000 vp per cell of Ad5/3 or Δ24RGD (Fig. 1B).

The engineered dysplasias showed less lysis when the effects of exposure to Δ24RGD or Ad5/3Δ24 were compared with dl922-947 (Table 1). There was no relationship between expression of ov integrins and lysis after exposure to Δ24RGD, although hexon and E1A were frequently detected in the upper and spinous layers, suggesting that these CRAds are able to infect dysplastic keratinocytes, but that their ability to replicate may be impaired when compared with dl922-947.

### Table 1. Comparison of the lytic effect of dl922-947, Δ24RGD, Ad5/3 and Adwt at 1, 10, and 100 vp per cell for normal, dysplastic, and malignant engineered oral epithelia 14 d after infection

<table>
<thead>
<tr>
<th>Cell type</th>
<th>dl922-947</th>
<th>Δ24RGD</th>
<th>Ad5/3</th>
<th>Adwt</th>
<th>Ki67 proliferation index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>NK</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SPDOK</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>LTDOK</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CDOK</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>D6</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>D20/KGF</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>D20/EGF</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>POE9n</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DOK</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>LDOK</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>JHU022</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE: The Ki67 proliferative index is also shown for the tissue equivalents generated using these strains (NK, normal nonkeratinizing mucosa; K, normal keratinizing mucosa). Visual scoring for CRAd infection: 1, occasional lytic cavities in the upper epithelium and some loss of polarity of the basal cells at day 14; score 2, areas of lysis in the upper layers extending to the spinous cells by day 14; score 3, lytic cavities in the upper epithelium with a mixed pattern of lysis in the lower layers with areas where all cells were lost mixed with foci where the basal cells remained at day 14; score 4, many lytic cavities throughout the epithelia at day 7 and complete lysis by day 14; score 5, complete lysis of the epithelium by day 7.

Phenotypic characteristics of the tissue-engineered dysplastic oral epithelia modulate the severity of lysis with dl922-947. The lysis score was not related to the severity of dysplasia in that following infection with dl922-947 at 100 vp per cell, epithelia derived from the JHU022 and LDOK strains dissolved as completely as the epithelia derived using the D6, D20 (maintained with EGF), and the DOK strains. However, the engineered dysplastic epithelia derived from LTDOK, POE9n, D20 (maintained with KGF), and SPDOK showed a mixed pattern of complete and partial lysis at the same dose level.

The tissue-engineered dysplastic oral epithelia were characterized by different proliferation indices and patterns of Ki67 expression (Table 1, Fig. 5A). In general, the dysplastic epithelia with the highest rate of basal and suprabasal proliferation showed the most efficient lysis after exposure to dl922-947. When the D20 epithelia was maintained with EGF, as opposed to KGF, more suprabasal mitoses were detected (Table 1), and after exposure to dl922-947 or Δ24, the lysis score (Table 1) and number of E1A spots was increased (Fig. 4A), supporting the view that the number of cycling suprabasal cells modulates the lytic response. However, this is not the only factor influencing the extent of lysis because some basal cells remained when the POE9n epithelia, which shows frequent suprabasal mitoses, was infected with dl922-947. The D6 engineered oral epithelia, with a low Ki67 proliferative index, also lysed completely (Table 1).

www.aacrjournals.org 7289 Cancer Res 2007; 67: (15). August 1, 2007

Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2007 American Association for Cancer Research.
The POE9n dysplastic epithelium was also infected with dl922-947 followed by exposure to Ad5/3Δ24 or Δ24RGD. The basal cells of this epithelia do not express CAR or significant levels of αv integrins, but a second round of infection with a CAR-independent vector increased the number of sites where lysis of basal cells occurred when compared with a single round of infection with dl922-947 (data not shown).

**Discussion**

We report that topical delivery of dl922-947 can dissolve tissue-engineered oral dysplasia, and that the normal counterparts are very resistant to lysis. The lytic effect of dl922-947 for oral dysplasia was significantly greater than that observed with d/1520 or CRAds modified to bind to alternative cellular receptors. Previous studies have shown that dl922-947 and Adwt can infect and replicate in

![Figure 3](image-url)

**Figure 3.** Lytic effect of dl922-947 and expression of viral hexon protein and E1A indicating that this CRAd is able to reach the deeper layers of the epithelium, replicate, and cause lysis in a dose-dependent manner. A, top row, destruction of the tissue-engineered D20 dysplastic epithelium after exposure to 1 and 100 vp per cell of dl922-947 showing superficial keratosis and the appearance of lytic cavities at day 5 at low and high power (LP, HP). The number of ghost cells and superficial debris increased over time such that there is significant thinning of the epithelium at day 12 and loss of all keratinocytes by day 14. Second row, similar treatment effects after exposure of the engineered DOK dysplastic epithelium to 10 and 100 vp per cell. Third row, lytic cavities developing for the tissue-engineered POE9n and D6 dysplastic epithelia after exposure to 100 vp per cell. Rapid lysis of the engineered LDOK epithelium is also shown. B, representative tissue-engineered dysplastic oral epithelia showing expression of hexon for the D20 and LDOK strains at day 4 following infection with 1 and 10 vp per cell of dl922-947, respectively. As the dose of virus is increased, hexon protein is detected in the lower epithelial layers. Infection of the engineered LDOK epithelium with 10 vp per cell produced complete dissolution, and increased expression of hexon protein is shown at days 1 and 7 as viral replication and lysis progressed. In contrast, the POE9n dysplastic epithelium showed a focal pattern of hexon expression at day 7 following exposure to 100 vp per cell of dl922-947. C, expression of E1A was dose dependent, and examples are shown at days 1 and 4 for the engineered LDOK epithelia after infection with 1 and 10 vp per cell of dl922-947, for the D20 dysplastic epithelium at day 7 after exposure to 10 vp per cell, and for engineered D6 epithelia at day 7 after exposure to 10 and 100 vp per cell.
clinical lesions after 48 h (20), but explants of normal and dysplastic oral mucosa underwent rapid autolysis, and we were only rarely able to maintain these tissues over this time frame. Recognizing these limitations, we developed an in vitro system to tissue engineer normal and dysplastic epithelia showing mild, moderate, and severe dysplasia using keratinocytes derived from normal tissues and clinical lesions. These tissue equivalents showed different rates of proliferation, subrabasal mitoses, disordered keratin expression, and increased expression of the EGF receptor and E cadherin when compared with normal tissues, mimicking the in vivo situation. The engineered epithelia are comprised of different numbers of viable cell layers, but lack rete ridges that are a feature of clinical lesions. The immortal dysplastic oral epithelia and the normal counterparts also showed a modest increase in the Ki67 proliferation index when compared with patient-derived tissues (29) due to the requirement to add exogenous growth factors to maintain tissue viability (30). Most mortal engineered epithelia had a very low proliferative index, likely reflecting the inherent tendency of these cells to differentiate and eliminate abnormal cells from the epithelium in vivo and our inability to define growth conditions that could support continued proliferation of these cells. However, in all other respects, these engineered dysplastic epithelia resemble the clinical lesions remarkably closely and, together with the normal counterparts, provide a new resource for assessing the efficacy of novel approaches for the treatment of oral precancer.

We anticipated that the grade of the dysplasia would be the most important factor influencing lysis, but this was not the only parameter modulating the lytic effect, and we examined other tissue characteristics that we considered might affect the response to d922-947. Replication of CRAds occurs in cycling cells, and we anticipated that we might see lysis of the basal layer of engineered normal oral epithelia, but few lytic foci were observed. The proliferation index for the basal and suprabasal compartments of most mortal engineered dysplastic epithelia was low, although the strain with the most cycling basal cells showed a high lysis score after exposure to 100 vp per cell. We also found that increasing the number of suprabasal cycling cells, by maintaining the D20
dysplastic epithelia with EGF as opposed to KGF, increased the lytic effect. However, the POE9n epithelia, with a high basal proliferation index, showed only patchy basal cell lysis following infection with dl922-947, indicating that whereas the number of replicating cells is important, other factors influence the treatment response.

Expression of key proteins influencing senescence and the G1-S checkpoint (31–33) might also be important as keratinocytes lacking p16 and those with high levels of RbP cycle more frequently, and this may support more efficient replication and lysis. However, the engineered mortal dysplasias that expressed p16, low levels of RBp, and wild-type p53 showed complete lysis after exposure to dl922-947 or Δ24. This suggests that the lytic effect of these CRAds may not necessarily be dependent of the loss of these critical regulatory proteins or p53 gene status.

The finding that the mortal dysplastic epithelia lysed as efficiently as their immortal counterparts prompted us to investigate expression of p300, a cofactor that interacts with E1A to induce S phase (34). We anticipated that expression of p300 would be associated with differentiation (35), but this cofactor was widely expressed in normal and dysplastic epithelia, including those with a relatively undifferentiated phenotype.

Based on these observations, we propose that nonkeratinized normal oral mucosa resists lysis with dl922-947 because the multilayered nature of the epithelium, with abundant tight junctions, prevents any virus reaching the proliferative basal cells. When the epithelium is keratinized, the superficial layers provide an additional barrier, but if CRAds reach the spinous layers, replication might occur in cycling cells, or retention of the CR1 region in dl922-947 may stimulate S phase in postmitotic cells (36) producing focal lysis, and this effect was confirmed using our in vitro systems.

The engineered dysplasias express CAR on a proportion of the upper cell layers, and exposure of dysplastic keratinocytes to

---

**Figure 5.**Demonstration of the tissue characteristics that modulate the lytic response. Representative examples of (A) Ki67 staining show a predominantly basal pattern for the tissue-engineered D20 epithelia maintained with KGF and DOK, whereas the POE9n epithelia had many suprabasal proliferating cells. B, the CAR was expressed throughout the epithelia for nonkeratinized buccal mucosa but restricted to the lower layers at keratinized sites. The typical pattern was recapitulated for tissue-engineered normal and dysplastic oral epithelia prepared using the D20, DOK, and POE9n strains. The engineered DOK dysplastic epithelia showed weak expression on the upper epithelial layers and the POE9n strain sites where this receptor was absent on the basal cells, a pattern that was also found for some clinical dysplastic lesions (D1, D2). C, expression of the cofactor p300 was restricted to the basal layer at nonkeratinized sites, but found throughout the epithelial layers for keratinized normal oral epithelia, the matched parakeratinized tissue-engineered normal oral epithelium, and the CDOK mortal dysplastic epithelium. Most clinical dysplastic lesions expressed p300 throughout the epithelia (D3–D5), although some tissues (for example, D6) showed a mixed pattern with adjacent positive and negative areas.

---

Cancer Res 2007; 67: (15). August 1, 2007 7292 www.aacrjournals.org
d922-947 increased the proportion of cells in S phase as previously reported (37), but this response was not seen when normal keratinocytes were exposed to this CRAd. Thus, we hypothesize that following the infection of dysplastic keratinocytes with d922-947, S phase induction occurs due to the retention of the CRE region, expression of p300, or aberrations affecting the cell cycle or apoptosis that promote viral replication, and that these effects contribute to the superior potency of d922-947 when compared with Adwt. The high proliferation index and frequent suprabasal mitoses that characterize the immortal dysplasias, activation of adeno-virus early promoters as differentiation occurs (E1A, E2A, and E4; ref. 38), together with the altered mechanisms for RNA export that promote viral replication (39), may also facilitate productive viral DNA amplification.

The location of E1A staining suggested that replication commenced at the sites where CAR is abundant and the progeny released following cell rupture enter adjacent cells using this receptor. The increased permeability of dysplastic epithelia compared with normal mucosa (40), or changes in cell shape and adhesion that occur during infection, may also help the virus to pass into the deeper tissues by a combination of repeat cycles of infection and replication. The virus may also infect oral keratinocytes via alternate primary receptors including perlecan (41) and the α2 domain of the MHC (42). We found that some dysplastic epithelia do not express CAR on the basal cells, and this may explain why the lysis of the POE9m epithelia is largely confined to the upper layers. Low levels of E1A were detected in the basal keratinocytes that remained, strongly suggesting that if left for longer periods of time, complete lysis might occur.

We investigated whether Δ24RGD or Ad5/3Δ24, vectors that overcome any CAR deficiency (43–45), could lyse engineered dysplastic epithelia more efficiently, but found that these CRAds produced less lysis than d922-947 or Δ24. The most likely explanations for this are that levels of Ad3 receptors are not as high on oral dysplasia as found for squamous tumors (13), and that intergrin expression is not sufficient to promote targeting and internalization of Δ24RGD. We found that some tissue-engineered dysplastic epithelia showed complete lysis after exposure to a single dose of d922-947, whereas foci of basal cells remained when the POE9m and the SPDOK and LTDOK strains of mortal oral keratinocytes were exposed to this CRAd. It is to be anticipated that similar partial therapeutic effects would be seen in the clinic, but that repeat treatment with the same vector and developing protocols incorporating CRAds that infect cells using alternative primary receptors will help to maximize destruction of these abnormal keratinocytes. Ongoing improvements in vectorology that enhance the infectivity of these viruses (46–50) and exploit differences between normal and dysplastic oral keratinocytes, for example, increased expression of cell surface extracellular matrix components to further augment this process, may also be beneficial. However, effective destruction of all dysplastic keratinocytes will probably depend on developing strategies that exploit the knowledge of the pathways that sustain the suprabasal and increased proliferation of these clones, and the effect of combining the lytic effect of CRAds with small molecules that modulate epithelial proliferation can be tested in future studies.

Many patients prefer to have a precursor lesion removed to reduce the requirement for long-term follow-up to reduce the anxiety about the diagnosis and risk of tumor development. We have shown that d922-947 has the potential to dissolve these lesions. This treatment may also be able to eliminate oral mucosa that looks normal on visual inspection, but is genetically and phenotypically abnormal, adding a degree of biological selectivity to the management of oral dysplasia.

Acknowledgments


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

We thank Dr. John Harrison for help with evaluating the morphology of these cultures and Dr. Kate Rauen for the kind gift of the CAR 72 antibody.

References

21. Partridge M, Green MR, Langdon JD, Feldmann M. Production of TGF-α and TGF-β by cultured keratinocytes, skin and oral squamous cell carcinoma—potential autocrine regulation of normal and
41. Baraschi T, Yonezohori H, Ohoshi K, Cheng J, Saku T. Intraepithelial expression of perlecan, a basement membrane-type heparan sulphate proteoglycan reflects
Lysis of Dysplastic but not Normal Oral Keratinocytes and Tissue-Engineered Epithelia with Conditionally Replicating Adenoviruses

Kamis Gaballah, Allison Hills, David Curiel, et al.