Experimental evidence supports the abscess theory of development of radicular cysts

Nair, P N R; Sundqvist, G; Sjögren, U
Experimental evidence supports the abscess theory of development of radicular cysts

Abstract

OBJECTIVE: The objective of this study was to experimentally induce inflammatory cysts in an animal model so as to test the hypothesis that radicular cysts develop via the "abscess pathway."

METHODOLOGY: Twenty-eight perforated custom-made Teflon cages were surgically implanted into defined locations in the back of 7 Sprague Dawley rats. A week after the implantation of the cages, a known quantity of freshly grown, close allogeneic oral keratinocytes in phosphate buffer solution (PBS) was injected into each cage. One cage per animal was treated as the control that received only epithelial cells. The remaining 3 cages of each animal were trials. Seven days post epithelial cell inoculation; a suspension of 0.2 mL of Fusobacterium nucleatum (10^8 bacteria per mL) was injected into each of the 3 trial cages. Two, 12, and 24 weeks after the inoculation of the bacteria, the cages were taken out, and the tissue contents were fixed and processed by correlative light and transmission electron microscopy. Sixteen of the 21 trial cages could be processed and yielded results. RESULTS: Inoculations of epithelial cells followed 1 week later by F. nucleatum into tissue cages resulted in the development inflammatory cysts in 2 of the 16 cages. The 2 cages contained a total of 4 cystic sites. None of the control cages showed the presence of any cyst-like pathology. CONCLUSIONS: Inflammatory cysts were induced by initiating acute inflammatory foci (abscess/necrotic area) by bacterial injection that got enclosed by a proliferating epithelium. This finding provides strong experimental evidence in support of the "abscess theory" of development of radicular cysts.
Experimental evidence supports the abscess theory of development of radicular cysts

P.N.R. Nair, BVSc, DVM, PhD (Hon), Göran Sundqvist, DDS, PhD, and Ulf Sjögren, DDS, PhD

a. Institute of Oral Biology, Center of Dental and Oral Biology, University of Zurich, Zurich, Switzerland
b. Department of Odontology, Umeå University, Umeå, Sweden

Address for Correspondence:

Dr. P.N.R. Nair, Center of Dental & Oral Medicine, Abteilung für OSD, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland
Phone: 0041-44-634 31 42, Fax: 0041-1-312 32 81, E-mail: nair@swissonline.ch
Abstract

Aim. To experimentally induce inflammatory cysts in an animal model so as to test the hypothesis that radicular cysts develop via the ‘abscess pathway’.

Methodology. Twenty-eight perforated custom-made Teflon cages were surgically implanted into defined locations in the back of 7 Sprague Dawley rats. A week after the implantation of the cages, a known quantity of freshly grown, close allogenic oral keratinocytes in phosphate buffer solution (PBS) was injected into each cage. One cage per animal was treated as the control that received only epithelial cells. The remaining three cages of each animal were trials. Seven days post-epithelial cell-inoculation; a suspension of 0.2 ml of Fusobacterium nucleatum \( (10^8 \text{ bacteria per ml}) \) was injected into each of the three trial cages. Two, 12 and 24 weeks after the inoculation of the bacteria, the cages were taken out, the tissue contents were fixed and processed by correlative light and transmission electron microscopy. Sixteen of the 21 trial cages could be processed and yielded results.

Results. Inoculations of epithelial cells followed one week later by \textit{F. nucleatum} into tissue cages resulted in the development inflammatory cysts in 2 of the 16 cages. The two cages contained a total of 4 cystic sites. None of the control cages showed the presence of any cyst-like pathology.

Conclusions. Inflammatory cysts were induced by initiating acute inflammatory foci (abscess / necrotic area) by bacterial injection that got enclosed by a proliferating epithelium. This finding provides strong experimental evidence in support of the ‘abscess theory’ of development of radicular cysts.

Keywords Apical abscess, apical periodontitis, dental cysts, inflammatory cysts, periapical cysts, radicular cysts, Cyst genesis.
Periapical or radicular cysts are inflammatory jaw cysts of teeth with infected and necrotic pulps. The cysts are a direct sequel to chronic apical periodontitis. But every apical lesion does not develop into a cyst. About 52% of all apical periodontitis contain proliferating epithelium,\(^{(1)}\) widely held to be derived from the cell rests of Malassez.\(^{(2, 3)}\) Although the reported prevalence of cysts among apical periodontitis lesions varies from 6 to 55%\(^{(4)}\) investigations based on meticulous serial sectioning and strict histopathological criteria\(^{(1, 5, 6)}\) show that the actual prevalence of the cysts is well below 20%.

There are two distinct categories of radicular cysts namely, those containing cavities completely enclosed in epithelial lining (true cysts) and those containing epithelium-lined cavities that are open to the root canals (pocket cysts).\(^{(1, 5)}\) More than half of the cystic lesions are apical true cysts and the reminder is an apical pocket cyst.\(^{(1, 5)}\)

Many authors attempted to explain the pathogenesis of apical cysts.\(^{(7-14)}\) The formation of the cysts has been described as taking place in three stages.\(^{(15)}\) During the first phase the dormant epithelial cell-rests proliferate, probably under the influence of inflammatory cytokines and growth factors\(^{(16-19)}\) that are released by various cells residing in the apical lesion. During the second phase an epithelium-lined cavity comes into existence, the mechanism of which is unknown. During the third phase, the cyst grows, the exact mechanism of which is also not fully understood. The theory based on osmotic pressure\(^{(20-22)}\) of cyst growth has receded to the background in favour of a molecular basis for the expansion of radicular cysts.\(^{(23-27)}\)

The biological basis of the second stage of cystgenesis, namely, the cavitation has been a point of contention for the past several decades. There have been two long-standing concepts regarding the formation of the cyst cavity. (1) The concept of central necrosis\(^{(10, 11)}\) of the epithelium assumes that the central cells of the epithelial mass get removed from their source of nutrition and undergo death and degeneration. The products in turn attract neutrophilic granulocytes into the necrotic area. Such micro cavities containing degenerating epithelial cells, infiltrating leukocytes and tissue exudates coalesce to form the cyst cavity lined by stratified squamous epithelium. (2) The abscess theory\(^{(28)}\) rests on the assumption that the proliferating epithelium surrounds an acute inflammatory focus (abscess) or necrosed connective tissue segment\(^{(11)}\) because of the innate nature of epithelial cells to cover exposed connective tissue surfaces. Recently it has been suggested\(^{(17)}\) that the proliferating epithelial strands may merge three-dimensionally, ‘trapping’ islands of inflamed connective tissue that degenerates due to a “loss of blood supply” to form eventually the cyst cavity. Although the epithelial tissue has no vasculature, the inflamed periapical connective tissue, except for abscessed/necrotized
areas, is well vascularized. It is quite unlikely that an area of inflamed but vital periapical soft connective tissue would be strangulated (17) or thrombosed (11) to death by the proliferating epithelium. Therefore, in the absence of any evidence the suggestion of connective tissue strangulation by epithelial strands has to be considered as biologically untenable.

Today, there is no evidence in support of any of the theories on cystgenesis. The aim of this study was to experimentally induce inflammatory cysts in an animal model so as to test the hypothesis that radicular cysts develop via the abscess pathway.

MATERIAL & METHODS

Approval to conduct the study was granted from the local institutional Research Ethics Committee.

Animal model

The experimental induction of inflammatory cysts was attempted in a ‘tissue cage’ model in Sprague Dawley rats weighing 300g to 500g. The cages consisted of perforated sterile cylinders of Teflon with an internal volume of 0.8 ml that were implanted subcutaneously into the back of the animals as described in earlier studies. (29-31)

Epithelial cells

The epithelial cells used in this study were obtained from palatal gingival biopsies of one experimental rat that was killed for the purpose. Autogenous or isogenic epithelial cells could not be used in this study due to technical reasons. The sites of biopsies were scrubbed with sodium hypochlorite (0.5%). In order to obtain sufficiently large number of epithelial cells several small biopsies were dissected out and briefly rinsed with freshly prepared Dulbecco’s phosphate buffer solution (PBS). Most of the subepithelial connective tissue was carefully removed from the biopsies. Thereafter, the biopsies were transferred into a tube containing alpha-minimum essential medium (MEM, Gibco, Life Technologies Ltd; Paisly PA4 9RF, U.K.) enriched with 10% fetal calf serum (Gibco) and kept in an ice-bath. The biopsies were then washed 10 times in PBS. Streptomycin sulphate (Sigma, St. Louis, Missouri, 63178 USA) and Gentamycin sulphate (Sigma) were added to get a final concentration of 74 U/ml and 3.4U/ml respectively. The biopsies were then subdivided into smaller pieces, immersed in a bottle containing 5 ml 0.25% Trypsin (Sigma) and kept agitating in a water bath. The supernatant was removed every 30 minutes (four times) and was replaced with fresh Trypsin solution of the same concentration. The composite supernatants were centrifuged (800rpm = 70xg) for 5 minutes after adding 5 ml Trypsin-inhibitor (1.5mg/ml; Sigma). The pellet was washed consecutively with Trypsin-inhibitor and keratinocyte growth medium (KGM, Promo Cell, Heidelberg, Germany), re-suspended in
KGM and seeded out in Primaria cell culture flasks (BD Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing KGM in a CO₂ incubator at 37°C. The cultivating medium was replaced every third or fourth day. After 12 weeks of incubation the cells were harvested. The epithelial cells were suspended in a freshly prepared cell-freezing medium containing 40% alpha-MEM, 50% fetal calf serum, 5% glycerol and 5% dimethylsulphoxide. The cells were deep frozen in small fractions and stored at a temperature of –135°C. At the time of experimentation the frozen cells were thawed and re-cultivated in Primaria cell culture flasks containing KGM. A total number of 140'000 to 190'000 epithelial cells harvested from the culture were injected into each cage as described below.

**Microorganisms**

The strain NCTC 10562 (National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London) of *Fusobacterium nucleatum* was used in the experiments. The bacteria were grown on Brucella blood agar (3% laked horse broth) in an anaerobic box with an atmosphere of 5% carbon dioxide and 10% hydrogen in nitrogen. The cells were harvested, washed twice in PBS, suspended in PBS, the cell concentration was adjusted to approximately $10^8$ bacteria per ml and the bacterial suspension was homogenized in a glass mortar before used in the experimental infections. All steps of bacterial processing were done in the anaerobic box.

**Experimental procedures**

A total of 28 cages were surgically implanted into predefined locations in the back of 7 rats. The animals were obtained from a breeder producing experimental rats of narrow genetic diversity (1 control + 3 Trial cages per animal). Seven days after the implantation, when the cages were lined by rich vascular tissues, the fluid of the tissue cage was aspirated and 0.2 ml of an epithelial-cell suspension was injected into all cages. The cells were suspended in PBS with an adjusted concentration of about $7 \times 10^5$ to $9.5 \times 10^5$ cells per ml. One cage per animal was treated as the control cage that was inoculated with epithelial cells only. The remaining three of each animal were treated as the trial cages. Seven days post epithelial cell-inoculation; 0.2 ml of a suspension of *F. nucleatum* $(10^8$ bacteria per ml) was injected into the three test cages. The bacteria were inoculated into the cages within 30 minutes of harvesting. The number of viable cells in the suspension was determined by serial dilution and cultivation on blood agar. Two, 12 and 24 weeks after the inoculation of bacteria, the cages were taken out, immediately immersed in half-strength Karnovsky’s solution, (32)
carefully opened, the tissue contents of the cages were removed and further fixed for several days before histo-morphological evaluation was done.

**Tissue processing for light microscopy**

The tissue cage specimens were subdivided into 0.5 to 1 mm slices using a sharp razor blade, and a Wild M-5 stereomicroscope (Wild, Heerbrugg, Switzerland), washed in 0.185 M sodium cacodylate buffer (pH 7.4; 360 mosmol), post fixed in 1.33% OsO\textsubscript{4} buffered in 0.067 M s-collidine (2,4,6 trimethylpyridine), dehydrated in ascending grades of ethanol and embedded in Epon\textsuperscript{®} (Fluka AG, Buchs, Switzerland). Survey sections of 1-2 µm thickness were prepared using glass or histodiamond knives (Diatome, Bienne, Switzerland) and an ultramicrotome Reichert OM-U2 (Leica, Glattbrugg, Switzerland), stained in periodic acid-Schiff and metyleneblue - Azur II and photomicrographed in a Dialux 20 photomicroscope (Leica, Glattbrugg, Switzerland) equipped with the digital camera Progress C14 (Jenoptik, Eching, Germany) and a digital imaging system (ImageAccess, Imagic, Glattbrugg, Switzerland). Serial step cutting was adopted to create suitable sites for electron microscopy.

**Correlative transmission electron microscopy (TEM)**

The survey sections were thoroughly studied in a light microscope and on the photomicrographs that were taken to identify suitable sites TEM. Epon\textsuperscript{®} blocks were first modified using the machine Leica EMTrim (Leica Microsystems) by preparing miniature pyramids of about 0.1-0.2 mm height at the site. The trimmed specimens were thin sectioned to a thickness of 60-80 nm using diamond knives (Diatome) in the ultramicrotome Reichert OM-U2 (Leica). The thin sections were collected on copper grids and double contrasted using lead and uranium salts. Therefore, the ultra-sections were washed in distilled water, dried and examined using the transmission electron microscope EM 400T (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 60 kV.

**RESULTS**

**Trial specimens**

These were 16 tissue cages that received separate inoculations of epithelial cells followed one week later by *F. nucleatum* as described already. Due to technical reasons 16 of the 21 initially implanted trial cages could be processed of which one, seven and eight cages had observation periods of two, 12, and 24 weeks respectively.

Two weeks after the inoculation of the bacterial suspension (i.e. three weeks post-epithelial-cell injection) one cage was found positive for the development of cystic lesions. The cage revealed three separate cyst-like lesions. One of them showed a distinct focus of acute inflammation with
disintegrating neutrophils that was two-third surrounded by a stratified squamous epithelium (Fig 1). The other two abscess foci (Fig 2) were completely enclosed in stratified squamous epithelia and were located about 0.5 to 1 mm away in the long axis of the cage from the one illustrated in Fig 1. The epithelial linings of the abscess foci sharply delimited the abscess from the surrounding soft connective tissue. The latter consisted of delicate fibrous tissue, numerous fibroblast-like cells, blood vessels and occasional nerve fiber bundles. Step-cutting and limited serial sectioning revealed that the epithelium lined lesion illustrated in Fig 1 was independent of the two foci illustrated in Fig 2 with intervening soft connective tissue. However, due to technical reasons it was not possible to clarify whether the two foci in Fig 2 were connected with each other or not. TEM examination of the ‘cyst wall’ (Fig 3) revealed a typical multilayered stratified squamous epithelium that clearly delineated the fibrous soft connective tissue from the necrotic and abscessed area. High-resolution TEM imaging of the interface between the epithelium and the connective tissue (Fig 4) revealed a typical basal lamina that ran parallel to the contour of the basal membrane of the basal epithelial cell. The abscessed area (Fig 3) showed a dense accumulation of completely disintegrated neutrophils and tissue remnants that contained several clusters (Fig 5) and free floating bacterial cells, presumably belonging to the species *F. nucleatum*.

One of the seven cages in the 12 weeks of observation was positive for the development of cyst-like lesions with morphological features similar to those described for abscess foci with complete epithelial enclosure. None of the eight trial cages in the 24 weeks of observation revealed any cyst-like pathology. All of them contained well vascularised fibrous soft connective tissue populated with fibroblast-like cells. Occasionally a very limited and localized infiltration of chronic inflammatory cells could be observed. Presence of adipose tissue was a consistent observation in the 24 week-cages.

**Control Specimens**

Due to obvious reasons positive controls could not be provided for this investigation. Negative controls consisted of seven cages that received inoculations of epithelial cells only. These control cages were also implanted (one each) into the back of the seven animals as described already. No cystic lesions were observed in any of the 7 control cages in the three observation periods. Limited proliferation of epithelial cells into an epithelial strand in non-inflamed connective tissue was observed in one cage at 2 weeks of observation. The transverse sections of the contents of the remaining 6 control cages revealed that the cages were filled with soft connective tissue consisting of fibroblasts, numerous blood vessels and occasional nerve bundles. Deposition of adipose tissue was a common feature in
the cages of six months of observation. As absence of something cannot be convincingly illustrated with validity in morphological works, no illustration of the absence of cystic lesions in control cages is presented.

In brief, two of the 16 trial cages were positive for the development of cyst-like lesions while all the 7 control cages remained negative.

DISCUSSION

This was a prospective, hypothesis-driven, experimental investigation that resulted in successful induction of inflammatory cysts in a murine model. The outcome provides strong evidence in support of the ‘abscess theory’ of development of radicular cysts.

The application of the tissue cages facilitated the inoculation of epithelial and bacterial cell-suspensions into a clearly defined subcutaneous tissue of the host and retrieval of the intact caged tissue after the experimentation. For the analysis of the tissue specimens the precise technique of correlative light and TEM was used so that selected areas in the specimens could be traced in stages from naked eye-level observation to high resolution details. This enabled to study the fine structure of various cells, epithelium-connective tissue interface and convincing observation of microorganisms in the abscess foci.

To our knowledge there does not seem to exist in the literature any previous report on a hypothesis-based experimental investigation that resulted in induction of inflammatory cysts. Valderhaug (35) used the method of Kakehashi et al (36) and induced apical periodontitis in simian teeth. Leaving the root canals of the monkey-teeth open to the oral environments and deliberately disregarding any aseptic precautions during the clinical procedures resulted in the induction of apical lesions. Periapical cysts developed in 11 of the 39 (28%) experimented teeth. The study, (35) however, did not resolve the long standing issue of the pathogenesis of radicular cysts because it was already known (36) that exposure of mammalian teeth to oral environments would lead to the development of apical periodontitis and some of them would be cysts. After extensive histological and light microscopic examination of the cysts, Valderhaug (35) concluded, “...there is no evidence in the material that cyst formation commences through an epithelial breakdown in the granuloma”. Although the illustrations of necrotized areas of apical granuloma enclosed in epithelium were convincing, there is no statement that the findings were more in support of epithelial cells lining an abscess focus. This probably influenced Shear to state as recently as 1992 (15) that the concept of epithelial breakdown in
apical lesions is the most “… widely supported theory” with “histological evidence”. However, Shear did not present any convincing evidence.

In the study reported here, inoculation of epithelial cells followed one week later by *F. nucleatum* into tissue cages resulted in the development of distinct inflammatory cysts in 2 of the 16 cages. The two cages contained a total of 4 cystic sites. Depending on whether a ‘tissue cage’ or a ‘cystic lesion’ is the unit of observation, the incidence of cysts could be 13% and 25% respectively of the 16 trial cages. There is convincing evidence that about 50% of all apical lesions are epithelialized but only less than 20% of all apical lesions are cysts.\(^1, 5, 6\) The prevalence of cystic lesions in the study reported here was well in agreement with studies based on serial sectioning of spontaneously occurring apical lesions in humans\(^1, 5, 6\) and also that of experimentally induced apical lesions in monkeys.\(^35\)

The results presented here show that inflammatory cysts developed only in trial cages that received separate injections of epithelial cells and *F. nucleatum*. But, none of the control cages that received only epithelial cells showed the development of cyst-like pathology, an observation that would with limitations discredit the theory of central epithelial necrosis.\(^10, 11\) This means that the establishment and proliferation of epithelium was more successful in trial cages, probably due to the influence inflammatory mediators and growth factors released during the inflammation\(^16, 18, 19\) as a consequence of the bacterial inoculation. Our additional, limited data (not included here) showed that pure cultures of *Actinomyces israelii* or a mixture of *Porphyromonas endodontalis* and *Peptostreptococcus micros* in association with epithelial cells resulted in the development of inflammatory cystic lesions. This means that injection of *F. nucleatum* and the other microbes into the tissue cages resulted in acute inflammation (abscess). The proliferating epithelium that presumably happened to be in the immediate neighbourhood of the abscess appears to have lined the vital connective tissue and enclosed the abscess focus. Two-dimensionally, the epithelium gave the impression of “ring-fencing” the abscess which in three dimensional reality would be “ball-coating” of it. These observations are in strong support of the abscess theory of cystgenesis.

Further, these results provide some clues about the relative low incidence of cysts among apical lesions. For the development of an inflammatory cyst, it seems that a number interrelated yet independent, structural, microbial and patho-physiological, factors should be simultaneously present at a particular tissue site. The prevalence of cysts in apical lesions, therefore, would be determined by
the statistical probability of all such factors occurring simultaneously at the site of initiation of a cyst in an apical periodontitis lesion.

The results of this study allow the conclusion that inflammatory cysts are most likely induced by the initiation of an acute inflammatory focus (abscess / necrosis) that gets enclosed and delimited by a proliferating epithelium. This finding provides strong experimental evidence in support of the ‘abscess theory’ of development of inflammatory periapical cysts.

Acknowledgements We thank Mrs. Chrissie Roth, Mrs. Margrit Amstad-Jossi and Mrs. Susy Münzel-Pedrazoli for excellent technical assistance.
Legends

Fig 1. Photomicrograph of a transverse section (a) of the contents of a rat tissue cage inoculated with a suspension of epithelial cells followed one week later by a suspension of *F. nucleatum*. Note the abscessed area (AB), incompletely but mostly enclosed by a stratified squamous epithelium (EP) and delimited from the healthy connective tissue (CT). The rectangular demarcated area in (b) is magnified in (c). The right and left demarcated areas in (c) are further magnified in (d) and (e) respectively. Note the flattened, elongated and stratified epithelial cells (EP) and the dense accumulation of disintegrating neutrophils of the abscess (AB) in (e). Original Magnifications: a x15, b x 22, c x55, d x220, e x920.

Fig 2. Light microscopic view of a transverse section of the contents of the rat tissue cage inoculated with a suspension of epithelial cells and *F. nucleatum*, one week after inoculation of the bacterial suspension. Note a pair well developed (arrow heads) inflammatory cysts in (a). The larger of the lesions in (a) is magnified in (b). The abscessed areas (AB) are completely enclosed and delimited from the healthy connective tissue (CT) by a stratified squamous epithelium (EP). The rectangular demarcated area in (b) is magnified in (c). The arrowed epithelial segment and the demarcated abscess area in (c) are further magnified in (d) and (e) respectively. Note the elongated and thinly flattened exfoliated epithelial cells (arrowheads) among a dense congregation of disintegrated neutrophils in the abscessed area (AB). Original Magnifications: a x15, b x 55, c x220, d x440, e x920.

Fig 3. Composite transmission electron micrograph showing subepithelial connective tissue (CT), stratified squamous epithelium (EP) of the cyst lining and the epithelium-enclosed abscess (AB) illustrated in Fig 2. Original magnification: x20’000.

Fig 4. High resolution transmission electron microscopic view of the epithelial-connective tissue interface. Note the electron lucent connective tissue (CT) and the electron dense basal epithelial cells (BC). A typical basal lamina (BL) runs parallel to the contour of the basal membrane of the basal cells (BS). Magnification x20’000.

Fig 5. Composite electron micrograph showing further magnification of the epithelium-enclosed abscess illustrated in Fig 2 and 3 respectively. The highly electron dense structures distributed throughout the micrograph are the nuclear remnants of disintegrated neutrophils. Note the filamentous bacterial clusters in the circular demarcated areas numbered C1 to C5. The microbes in C1 and C3 are further magnified in Fig 6. Original magnification: x2’200.
Fig 6. High resolution transmission electron micrographs of microbes demarcated in C1 (a) and C3 (b) in Fig 5. Note the tangentianally cut clusters of filamentous microorganisms (arrowheads). Original magnifications: x6'000.
REFERENCES


Figure 3

CT

EP

AB

Scale bar: 7 µm