Evaluation of the Cytotoxic and Genotoxic Effects of Orthodontic Bonding Adhesives upon Human Gingival Papillae through Immunohistochemical Expression of p53, p63 and p16

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Abstract. *Background: Numerous in vitro studies have shown that composite materials, commonly used for restorations in conservative dentistry, and in orthodontics to anchor brackets to the tooth enamel, have cytotoxic and genotoxic effects. The study determined expression of p53, p63 and p16, biomarkers useful for predicting potential genotoxicity. Patients and Methods: p53, p63 and p16 expression was determined immunohistochemically in the gingival papillae of 99 patients (69 banded orthodontically for at least one year, brackets bonded to teeth with filled flowable composite resin, 30 without orthodontic banding as controls). The papillae samples were removed surgically and examined to evaluate morphological and biological alterations. Results: In no case were morphological alterations visible by microscopy out of the 69 banded patients; four (5.80%) were positive for p53 and two for p63 expression in the basal and suprabasal layers (2.90%). One patient was positive for p16 (1.45%). No control case was positive for any of the biomarkers (0.00%). Conclusion: The significance of p53, p63 and p16 positivity, and whether these proteins may serve as biomarkers to predict the risk of developing oral lesions (dysplasia, oral cancer) is still unclear. Although details of the mechanisms leading to cell death, genotoxicity and cell-cycle delay are not fully understood, resin monomers may alter cell function in the oral cavity.*

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Numerous *in vitro* studies have shown that composite materials, in particular some monomeric adhesives used in dentistry, have cytotoxic and genotoxic effects on the cell and it has been established that the co-monomer triethylene glycol dimethacrylate (TEGDMA) causes gene mutations *in vitro* (1-15). Such materials are commonly used for restorations in conservative dentistry, and in orthodontics to anchor brackets to the dental enamel, the bracket being fixed to the vestibular surface of the tooth. Orthodontic adhesives may be subdivided into the following general categories: hybrid cements; vetroionomeric cements; self-curing resins and light-curing resins. The latter type are in most widespread use. In the first two categories, the polymerization process is activated chemically, while in the case of light-curing resins activation is physical and is produced by light.

The polymerization of orthodontic adhesives is never complete (due to the inhibitory effect of the presence of oxygen polymerization remains incomplete at the surface for an approximate thickness of 10-85 μm) (13) and up to 50% of the components do not participate in the reaction. This means that relatively large amounts (up to 14%) of non-polymerized and potentially toxic material may be released. The orthodontic adhesive in most widespread use at the Orthodontics Department of the University of Milan Dental Clinic is a light-cured composite resin (3M Unitek Transbond XT Light-cure adhesive) available in the form of a kit containing paste and liquid (primer) which is activated by visible light and uses *bis*phenol A dimethacrylate (BisGMA), BisGMA bis and TEDGMA as monomers.

In vitro studies to evaluate the cytotoxic effect of some of these orthodontic adhesives have shown that the adhesive (paste) used in orthodontics to anchor the brackets is less cytotoxic than the primer (liquid) (13). The differing cytotoxicity of the primer and paste is due to their different

chemical compositions: the liquid primer, which contains no fillers, has a higher concentration of monomers than the paste and thus releases greater quantities than does the latter (15). The toxicity of the monomers BisGMA, BisGMA bis, TEGDMA, hydroxy ethylimethacrylate (HEMA) and urethane dimethacrylate (UDMA) has been extensively investigated and numerous studies (9, 10) have shown them to be released by polymerized resins (16). There is also evidence that the components of the composite materials extracted in aqueous solution cause cytotoxic effects on cultures of primary and immortal cells (8-11). A recent study evaluated the *in vitro* cytotoxicity of these monomers on cultured human gingival fibroblasts and keratinocytes using Alamar Blue (a very sensitive technique to determine cell proliferation quantitatively); all the monomers showed toxic effects (9,10).

Other studies have evaluated chromosome aberrations by counting micronuclei induced in V79-4 cells (fibroblast cultures) and have found that monomers possess *in vitro* mutagenic activity (3, 12) and cause an increase in apoptosis (12). The mechanism underlying these alterations is related to increased oxidative stress (3, 14) induced by the monomers in the cell (4 6). Increased oxidative stress causes DNA damage that may be repaired, produce a mutation, block the cell cycle, or trigger apoptosis (14).

The present prospective study investigated the cytotoxic and genotoxic effects on cells in the oral cavity of orthodontic resins by examining the morphology of the gingival papillae of 99 patients under light microscopy and histochemically analyzing the expression patterns of the p53, p63 and p16 proteins (regulators of the cell cycle and biomarkers for oral lesions: dysplasia, oral cancer).

Patients and Methods

Between September 2006 and April 2008 at the Milan University Dental Clinic, 69 patients who had been undergoing fixed orthodontic treatment for at least 12 months (previous diagnosis of malocclusion in different classes, assigned to orthodontic treatment) were selected as the study group. Thirty patients not undergoing orthodontic treatment with no clinical signs of disease were enrolled as negative controls. Table I summarizes the clinical data. Gingival papillae samples were removed surgically from all the subjects under local anesthesia (20% mepivacaine with adrenaline 1:100000; needle 27G mounted on Carpule disposable syringe) including part of the underlying connective tissue, using a scalpel with no. 15 blade; biopsy dimensions were 3×2×1 mm. The papillae were fixed in formalin and sent to the Pathological Anatomy Laboratory of San Gerardo Hospital, Monza, Italy. The study was approved by the institutional review board, and written informed consent was obtained from all the patients and controls.

Tissue processing and immunohistochemistry. The tissue sections, mounted on slides and stored at room temperature, were processed within 4-6 weeks from sectioning to maintain their antigenicity. The avidin-biotin complex immunoperoxidase technique was used (17).

Table I. *Age and gender of subjects in the study.*

	Study group	Control group
N	69	30
Male (%)	34 (49.28%)	15 (50%)
Female (%)	35 (50.72%)	15 (50%)
Mean age±SD (range)	23±4 (13-32)	25±2 (15-31)

Immunohistochemistry was conducted with the following antibodies: mouse anti-human p16, clone E6H12, Novocastra, Newcastle, UK, dil. 1:20; anti p53, clone DO7, Neomarkers, Fremont, CA, USA, dil. 1:100; anti p63, clone 4A4, Neomarkers, dil. 1:50. Antigen unmasking of protein p53 was conducted by placing the slides, completely immersed in citrate buffer at pH 6, in a microwave oven set to 750 W for three min cycles. At the end of each cycle, fresh buffer was added to replace that lost by evaporation. At the end of the third cycle, the slides were allowed to cool for at least 20 min before staining. Antigen unmasking of protein p16 was similarly conducted, immersing the slides in Tris EDTA buffer at pH 9 with four min cycles in a microwave oven set to 750 W. No antigen unmasking procedure was required for p63.

For each antibody used, appropriate positive control slides were prepared with tissue that definitely contained the antigen (to verify the effectiveness of the antibody used); negative control slides were prepared omitting the primary antibody (to check the specificity of the reaction and signal intensity).

The p53 protein is normally absent from the pluristratified epithelium or is limited to the basal layer (18). In the pluristratified epithelium, p63 protein indicates stem cells (19) and is normally present in the basal layer, progressively degrading towards the more mature layers (20). The slides were compared with those of control papillae, considered as negative. p53 (Figure 1A) and p63 (Figure 1B) positivity were evaluated by examining both the number of stained nuclei and their localization in the upper 2/3 of the epithelium, as has been proposed elsewhere (21, 22). The p16 protein is not normally expressed in gingival papillae. It was evaluated as positive when nuclear staining with or without cytoplasmic staining was present in ≥10% of cells in the supra-basal layers (23) (Figure 1C).

Cytotoxicity. Histological examination of the specimens stained with H&E at the optical microscopy, 100-250 magnification, evaluated, as in other studies (24), the number of altered cells (binucleate cells, hyperchromic nuclei, cells with altered nucleus/cytoplasm ratio, pleomorphic cells) and the number of dead cells (cariolysis, apoptotic cells). One thousand cells were counted and the specimens were considered positive if the number of altered cells plus the number of dead cells ≥50.

All the slides stained with H&E and by immunohistochemistry were evaluated separately by two surgical pathologists (FA, ED). In cases of disagreement, the specimens were re-evaluated using a multiheaded microscope until consensus was reached.

Fluorescent in situ hybridization (FISH). FISH analysis was performed at the Department of Pathological Anatomy, Brescia University, on the sample from one patient who showed

immunohistochemical positivity for p16 and on five control samples with no immunohistochemical alterations. FISH was applied using probes (Vysis Inc. Downers Grove, IL, USA) labeling the *p16* region (9p21) and the respective chromosome 9. The *p16* gene probe spans approximately 190 kb and contains a number of genetic loci including *p16(INK4A),* while chromosome 9 was identified by a centromeric α-satellite probe, following the manufacturer's recommendations. The kit consists of directly labeled fluorescent DNA probes specific for the *p16* gene (Spectrum Orange) and for the sequence at the centromeric region of chromosome 9 (Spectrum Green). The nuclei were counterstained using 4,6-diamidino-2-phenylindole (DAPI). A mounting medium for fluorescence (Vectashield, Vector Laboratories. Inc. Burlingame, CA, USA) was used; this preserved the fluorescence for several months. Each sample was independently evaluated by two investigators (ER, ED) with a fluorescence microscope (Nikon Optiphot-2, Nikon Instruments Inc., Melville, NY, USA) equipped with selective filters for the fluorochromes used, in high power fields (HPF; magnification ×600). The FISH images were captured and processed using Genikon software (Nikon Instruments S.p.A., Florence, Italy). FISH signals, visible as fluorescent spots, were counted in at least 200 non-overlapping nuclei; the scoring system was based on the percentage of nuclei with altered signals, following the criteria used by Qian and colleagues (25) for tissue sections (Figure 1D).

Results

The mean duration of orthodontic treatment was 29 months (range 12-30 months). No specimens were positive for cytotoxicity (0.00%); 4 were positive for p53 protein (5.80%); 2 were positive for p63 protein (2.9%) and one was positive for p16 protein (1.45%). FISH analysis did not demonstrate any molecular abnormalities of *p16* (chromosome 9 and/or gene alterations) (Figure 1D) either in the single *p16* immunohistochemically positive case or in the 5 control specimens tested.

Discussion

Unlike the *in vitro* studies, the present study used cells directly taken from the oral cavity. Immunohistochemically, four cases were positive for p53, whose expression is indicative of damaged DNA; in two specimens p63 was altered, indicative of an increased proliferative process and in one case p16 was positive.

The *p53* gene that codes for the p53 protein is a transcription factor of the *p21* gene and is expressed both when damage is reversible and when it is irreversible. When the damage is reversible, it blocks the cell cycle in the late G_1-phase, enabling the cell to repair the damage. In this period of block, the values of p53 and p21 are elevated, but once the damage has been repaired and the block removed they return to normal. When the damage is irreparable, the p53 protein triggers transcription of pro-apoptotic genes and the outcome is the irreversible programming of apoptosis.

The p53 protein may also increase when there is increased transcription of the human gene locus INK4a/ARF, which codes for p16 and p14 proteins. Both proteins, together with p53, are involved in the process of premature senescence, *i.e.* in the early state of reversible or irreversible block of the cell cycle. Senescence is considered to be the major tumor suppression mechanism since in this phase the cell does not respond to proliferative stimuli. Senescence may be induced by activation of either of two pathways: the first involves the p16 protein, which competes with cyclin D at the bonding site with cyclin-dependent kinase 4 (CDK4), impeding phosphorilation of the retinoblastoma protein (Rb) due to the non-formation of the active complex cyclin D/CDK4. This causes an irreversible block of the cell cycle. The second pathway involves the p14 protein, which impedes the murine double minute 2 (MDM2) from degrading p53, maintaining it at high levels. The p53 protein thus activates transcription of the p21 protein that, deactivating the cyclin D/CDK complex, makes blockage of the cell cycle reversible.

The p63 protein has two isoforms: ΔNp63 and TAp63. The form most widely expressed in mature epithelium, above all in the basal layer, where it indicates active proliferation is ΔNp63. In this study, only two specimens were found to be positive for the p63 protein and it was not clear whether or not this was due to the cells being in the proliferative phase.

None of the specimens showed morphological signs of cytotoxicity, whether expressed in terms of the number of altered cells, binucleate cells, anomalous nuclei, hyperchromic nuclei, altered nucleus-cytoplasm relationship or the number of dead cells (cariolysis, apoptotic cells), nor were there any signs of displastic or neoplastic alterations.

The study found examples of normal p16 protein with altered p53 protein and no visible morphological changes to the cells; normal p16 protein and altered p53 and p63 proteins and no visible morphological changes to the cells (apoptosis), and altered p16 protein with p53 and p63 also both altered. In the first case, the values of p16 being normal, it may be ruled out that the cell had entered premature senescence. Thus, with elevated p53 values, any cell damage must be attributed to genotoxic factors, with consequent temporary blocking of the cell cycle (checkpoint $G_1 \rightarrow S$, caused by the increase in p53) so as to enable DNA damage to be repaired. It remains to be determined whether this block was due to an attempt by the cell to repair DNA damage, or whether the active proliferation was the expression of a reactive process. Moreover, since there was no visible morphological cell damage, such as apoptosis, it would seem to point to slight injury, of a degree such as to be reparable.

In the second case, again within normal limits for p16 values, but with altered values of p53 and p63 proteins, it may be hypothesized that the block induced by p53 and p16 did not function and that there was a replicative cell

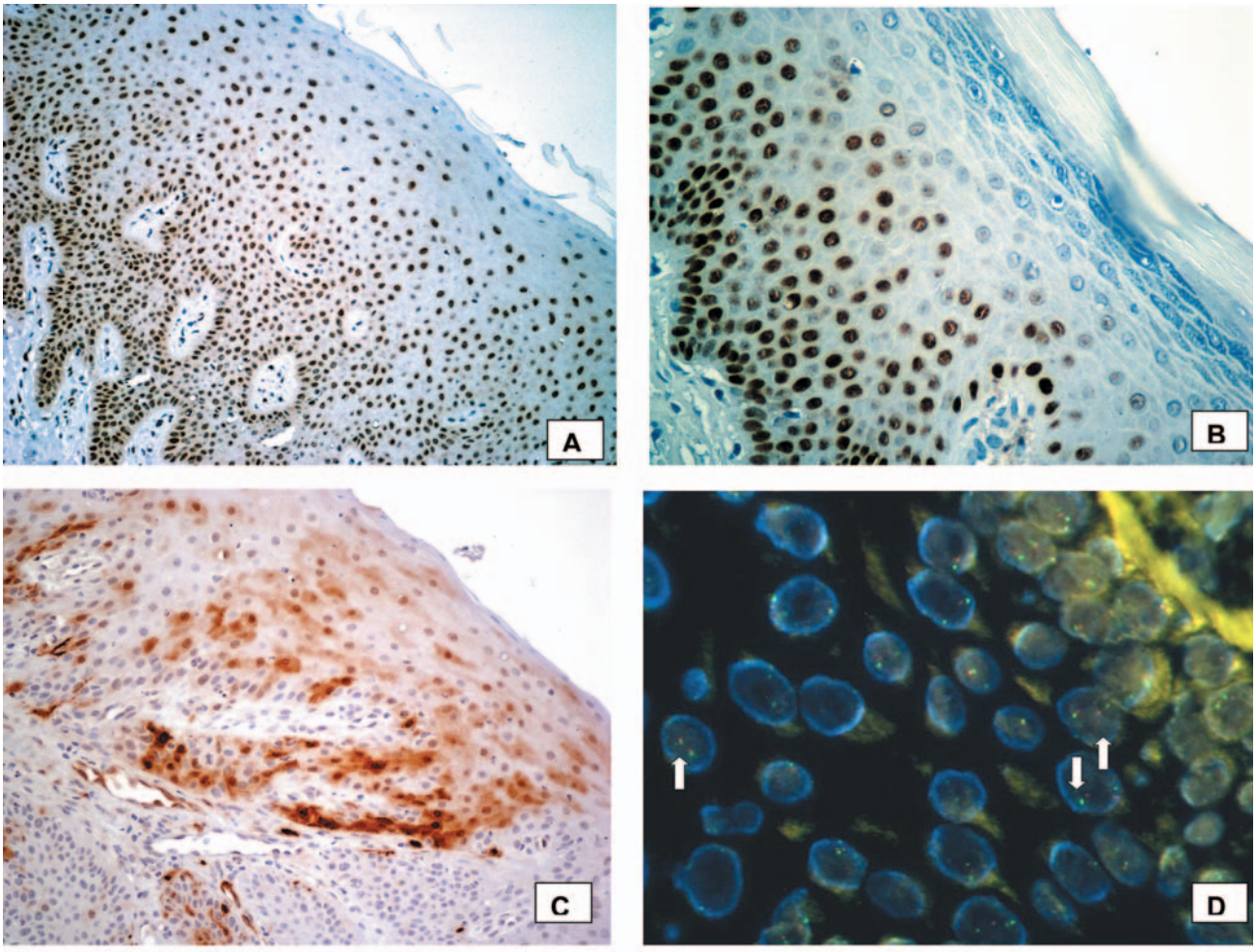

Figure 1. *Representative sections of one case. A, p53 nuclear positivity in all levels of epithelium (×200 magnification). B, Stained nuclei for p63 evident in the basal and medium level of the epithelium (×400 magnification). C, Nuclear and cytoplasmic expression of p16 (×200 magnification). D, Fluorescent in situ hybridation. Arrows indicate no alterations of p16 gene (red spots) nor of centromeric signal of chromosome 9 (green spots).*

response. In the third case, in which p16 was altered with altered expression of p53 and p63, it cannot be ruled out that a process of senescence may have been activated.

In summary, deviation from normal expression mostly concerned the p53 and p63 proteins, while the p16 protein was normal. In the only case found to be positive for p16, both p53 and p63 expressions were also altered, but there were no visible cell modifications, with a mechanism which could be like to the biomolecular pathway described by our group in the oral dysplasia (26) and in the bronchial cancerogenesis (27).

Thus the damage caused by the released monomers may be deduced to have induced DNA lesions that could not be repaired through cell cycle block and that the cells were actively proliferating, as shown by the p63 expression.

In conclusion, elevated values of the p53 and p63 proteins effectively demonstrates the genotoxicity of some adhesives used in orthodontics. Although details of the mechanisms leading to cell death, genotoxicity and cell-cycle delay are not fully understood, resin monomers may alter the functioning of the cells of the oral cavity.

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