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Preliminary results of immunohistochemistry, histology and of molecular biology on the healing mechanisms of periimplant soft tissues in contact with different titanium surfaces

Risultati preliminari di immunoistochimica e di biologia molecolare sui meccanismi di guarigione dei tessuti molli perimplantari a contatto con differenti superfici di titanio

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Summary

Introduction: during the healing process of wounds the restoration of the normal architecture and of the tissue function takes place through some fundamental phases like cellular migration through the matrix, the clot rearrangement, the epithelial migration, the formation of the basement membrane and the remodeling of the extracellular matrix (ECM). Among the various mechanisms involved, activation of proteases plays an essential role during the phases of cellular migration and tissue remodeling. Several systems are involved in the activation of the proteases among which the fibrinolytic one which is based on the activation of the plasminogen. The objective of this study has been to compare the biological behavior of soft tissues in contact with two different surfaces of titanium (sandblasted and laser treated) during the first phases of the periimplantar healing process. *Materials and Methods:* the healing model considered has been that of two migration areas of epithelial-connective tissues towards the center of the cover screws. Due to this a request has been made for the materialization of two different cover screws, one sandblasted and the other one laser treated. Biomolecular and morphological investigations have been made in order to find out if the healing processes of the fibromucosa, which goes to cover the cover screw meant as the neo-genesis of extracellular matrix, as well as proliferation of stromal cells and proliferation and migration of the covering epithelium, is influenced by the different methods of treatment of the metallic surface. *Results*: 2 male and 2 female patients have been enrolled. Each patient has received 4 implants inserted at crestal level for a total of 16 implants. 16 cover screws have been inserted of which 8 laser treated and 8 sandblasted (Table 1). 6 tissue samples have been taken for each patient at the established times for a total of 24 samples. Results obtained from studies of molecular biology, confirmed also by the morphometric investigations on immunohistochemistry, after the seventh day sampling, seem to suggest a greater expression of genes involved in the activation of proteolytic mechanisms of degradation of the collagenic and non-collagenic components of extracellular matrix in the tissue grown in contact with the laser treated surfaces as compared with those grown in contact with the sandblasted surfaces. *Conclusions:* these preliminary data, in spite of the limited number of patients and therefore of the samples taken, deserve confirmation by further studies with a larger sample of patients. Nevertheless, these data are to be kept in mind in the analysis of induction caused by different surfaces in titanium

on the healing mechanisms of soft peri-implantar tissues.

Key words: plasminogen system, uPAR, grip and go, implant surface, per-implants tissue healing.

Riassunto

Introduzione: *il sistema fibrinolitico con l'attivazione delle proteasi gioca un ruolo fondamentale durante i processi di guarigione delle ferite e quindi nel ripristino dell'architettura e della funzione tissutale. In ambito implantologico da diversi anni è stata introdotta la possibilità di modificare la superficie del titanio al fine di migliorare i processi di guarigione del tessuto osseo. In letteratura è ampiamente dimostrato come gli impianti ruvidi abbiano maggiori percentuali di successo rispetto a quelli lisci, tuttavia è meno studiato il comportamento dei tessuti molli (epitelio e connettivo) a contatto con tali superfici.*
L'obiettivo di questo studio è stato, attraverso l'analisi del sistema fibrinolitico, quello di confrontare il comportamento biologico dei tessuti molli a contatto con 2 differenti superfici di titanio, sabbiata e laserata, durante le prime fasi del processo di guarigione perimplantare. Materiali e Metodi: *Il modello di guarigione sperimentale sviluppato è stato quello di 2 fronti di migrazione di tessuto epitelio-connettivale al di sopra e verso il centro della vite tappo dopo l'inserimento dell'impianto. Sui campioni di tessuto prelevati sono state eseguite indagini di biologia molecolare di espressione genica (uPA; uPAR; PAI-1; MMP 2; MMP 9; TIMP-1 e TIMP-2) e analisi di immunoistochimica (uPAR+ e Ki67+ cells).* Risultati: *i risultati delle indagini di biologia molecolare, confermati anche dai risultati delle indagini morfometriche su immunoistochimica, hanno evidenziato a 7 giorni una maggiore espressione dei geni coinvolti nell'attivazione dei meccanismi proteolitici nei tessuti cresciuti a contatto con la superficie laserata. Il numero limitato e l'ampia variabilità tra i soggetti analizzati rende tuttavia le differenze non significative statisticamente.*
Conclusioni: *questi dati preliminari richiedono conferme da ulteriori studi con un maggior numero di campioni. Tuttavia sono da tenere in considerazione nell'analisi dell'induzione da parte delle differenti superfici in titanio sui meccanismi di guarigione dei tessuti molli perimplantari.*

Introduction

Connective tissues are made up of cells and of extracellular matrix consisting of an intricate net of macromolecules, mainly proteins and glucosamminoglicans on form of proteoglicans.
Variations in the relationship among the various macromolecular components and the way in which they are organized in the matrix explain the enormous diversity in structure, each one adapted to the functional requirements of a particular tissue.
During the healing process of wounds the restoration of the normal architecture and of the tissue function takes place through some fundamental phases like cellular migration through the matrix, the clot rearrangement, the epithelial migration, the formation of the basement membrane and the remodeling of the extracellular matrix (ECM). The extracellular matrix has an active and complex role in modeling the behavior of nearby cells influencing survival, development, migration, proliferation and function. The regulation of the "turnover" of macromolecules of the extracellular matrix is the crucial point for a great variety of biological processes. In some cases there is a rapid degradation, in others degradation has a more localized features. In everyone of these cases matrix components are degraded by extracellular proteolytic enzymes (proteases) which are excreted locally by the cells of the inflammatory microenvironment and by the epithelial cells.
Among the various mechanisms involved, activation of proteases plays an essential role during the phases of cellular migration and tissue remodeling. In fact some targets of these proteases are surface proteins among which the integrins involved specifically in the adhesion processes of the cells to the matrix.
Several systems are involved in the activation of the proteases among which the fibrinolytic one which is based on the activation of the plasminogen. Plasmin, active form of plasminogen,

degrades fibrin and transforms matrix pro-metalloproteases (pro-MMP) in active MMPs which in turn degrade the components of the extracellular matrix. The enzymes, which activate such system, are t-PU and the u-PA. The first is able to connect directly fibrin to the level of the clot, the second does not have site of connection for fibrin but for a specific surface receptor (u-PAR) expressed by numerous "types" of cells such as macrophages and monocytes.
Inhibition and regulation of the fibrinolitic system and of the MMPs takes place:
1) at the level of the physiological activators from the specific inhibitors of plasminogen (PAIs)
2) at the level of plasmin mainly due through the alpha 2 – antiplasmin
3) at the level of the MMPs due to the tissue inhibitors of the MMPs (TIPs 1 – 2)
The plasminogen system exercises a double role. One mediated by tPA, mainly involved in the homeostasis of fibrin, the other mediated by uPA mainly involved in the cell migration and remodellation.
Since the first description of the receptor of the urokinasic activator of plasminogen (uPA, urokinasis plasminogen activator, uPAR, uPA receptor), surface fibrinolysis has become one of the basis useful to the comprehension of invasive mechanisms and of cellular migration proteases dependent. In fact uPAR, when it connects to uPA activates the catalytic sites which leans towards the extracellular environment and is free to carry out its enzymatic activity which consists in the activation of plasminogen into plasmin. Plasmin is a serum proteases which degrades various molecules of the extracellular matrix but, most of all, activates pro-collagenases into active collagenases, capable to degrade collagens, which are the main components of matrixes and of anatomic barriers. Through this mechanism of proteolysis associated to the cellular surface, neoplastic cells, inflammatory leucocytes, germ epithelium, endothelial cells in the angiogenic mechanism, fibroblasts in tissue repair and so on, slide into tissue matrixes following the trail of the surface proteolysis. Furthermore uPA establishes adhesive relations with one of the most important adhesive molecules of the matrix, that is the vitronectin. Because cellular movement consists in a series of alternate adhesions to a substrate (the ground of the migration) and of the degradation of the same substrate (forward progression), the system uPA - uPAR has all the requisits needed for the cellular movement (grip and go). Consequently both isolated increase of uPAs and uPARs as well as a coordinated increase of both molecules cause an increase of cell migration properties.
In implantology several years ago has been introduced the possibility to modify the titanium surface in order to improve bone tissue healing processes. Addictive or subtractive treatments with modification in physical ad or chemical aspects of the implant surface have the purpose to obtain a roughness similar to that of bone surface due to the osteoclastic resorption action. The most common methods of surface treatment are sandblasting, acid etching, plasmaspraying and combinations. They produce an irregular topography with optimal dimensions but with a roughness that can be repeatable only if it is quantified statistically compared with other methods, such as laser treatment, which gives more repeatable surface roughness. Furthermore a problem, which is almost always due to superficial treatments, is the risk of surface contamination. The majority of the surface roughening techniques, be they subtractive of additive, cause implant contamination problems and the risk that on the surface may remain parts of products used during the surface treatments that may start dangerous corrosion actions with release of metallic ions with a possible negative effect on bone healing processes.
It has been amply demonstrated in literature that the rough implants have greater percentages of success than the smooth ones. Nevertheless, less attention has been given to the behavior of soft tissues (epithelium and connective) in contact with such surfaces.
The objective of this study has been to compare the biological behavior of soft tissues in contact with two different surfaces of titanium (sandblasted and laser treated) during the first phases of the periimplantar healing process.

Materials and Methods

After the development of the experimental model and of the protocol, the study has been submitted to the evaluation and authorization of the local ethical committee.

In the experimental model as support of titanium for the soft tissue study, has been choose cover screws that close the implant cavity after the positioning in the bone. The healing model considered has been that of two migration areas of epithelial-connective tissues towards the center of the screws. Due to this a request has been made for the materialization of two different cover screws, one sandblasted and the other one laser treated. Biomolecular and morphological investigations have been made in order to find out if the healing processes of the fibromucosa, which goes to cover the cover screw meant as the neo-genesis of extracellular matrix, as well as proliferation of stromal cells and proliferation and migration of the covering epithelium, is influenced by the different methods of treatment of the metallic surface.

Clinical Phase

- Ethical committee
- Cover screws (Geass and RTM Spa - Italy) have been requested with
  1) sandblasted surface with alluminium oxide with sand granules of 125µ at 100 mesh, with roughness of 1.2 µ in terms of RA
  2) surface with laser treatment DPSS ND:YAG in Qswitch regime with a roughness of 20 µ, step 30 µ and depth 10 µ.
- 4 patients have been selected and enrolled with the following criteria:
  - 2 males and 2 females
  - age between 40 and 65 years of age
  - recent and past medical history negative for systemic and local pathologies of odontostomatognatic interest
  - non smokers
  - edentuolism classification: total lower edentulism and lower intercalary endentulia, bilateral third class of kennedy
  - paraodontal indices in patients with intercalary edentulism: FMPS and FMBS less than 20%

Surgical Protocol

The patients have received 4 implants inserted at bone level so that the thickness of the repair soft tissues may not be excessive.

Independent on the implant diameter (3.8 , 4.5), a cover screw has been inserted to have in every patient 2 screws with laser- treated surface and 2 with sandblasted surface.

Suture has been carried out (two point of suture: one mesial and one distal to each implant) without covering totally the cover screws. This approach has been followed in order to have, during the healing process, two areas of connective epithelial migration starting from the margins towards the center of the screw.

Biopsy was taken with a mucotome of tissues when near the center of the cover screw.

Biopsy was taken in 3 steps

Two samples before inserting implants (T0 control): one sample on laser treated cover screw implant insertion site (third quadrant) and one sample on sandblasted treated cover screw implant insertion site (fourth quadrant).

Two samples at partial maturation (T1 – 7 days): one sample on laser treated cover screw implant insertion site (third quadrant) and one sample on sandblasted treated cover screw implant insertion site (fourth quadrant).

Two samples at complete maturation (T2 – 30-40 days: one sample on the other laser treated cover screw implant insertion site (third quadrant) and one sample on the other sandblasted treated cover screw implant insertion site (fourth quadrant).

Laboratory Phase

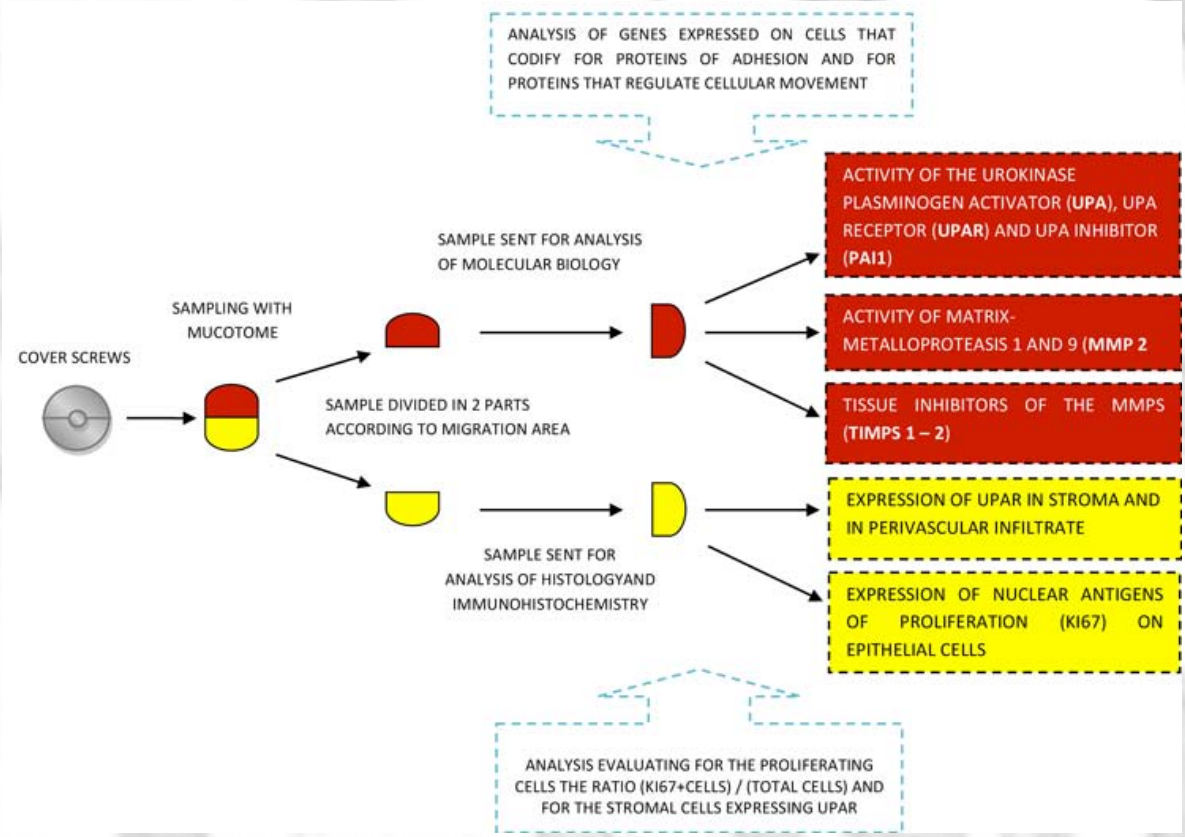
Analysis have been executed at the University of Florence:

A) Molecular biology has been carried out at the Department of Experimental Pathology and Oncology, University of Florence, Florence, Italy
B) Histology and immunohistochemistry at the Department of Anatomy, Histology & Forensic Medicine, University of Florence, Florence, Italy

Samples at our disposal where as follows:

- two control samples of healthy tissue taken from two of the sites receiving implants. Every sample has been divided into 2. One part has been sent for study of molecular biology and the other for studies of histology and immunohistochemistry;
- two samples for every one of the two subsequent phases of sampling. Every sample of epithelium-connective tissue taken above the two different cover screws, laser treated and sandblasted, was composed of two parts that is form the two areas of migration towards the center of the cover screw. Every sample has been divided into two according to the area of migration. One part has been sent for studies of molecular biology and the other for studies of histology and immunohystochemestry (Fig 1).

Figure 1. Each sample has been subdivided according to the area of migration in 2 specimens, one to be sent for studies of molecular biology and one for histological and immunohistochemical analysis



A) Analisys of molecular biology

These analysis have been carried out using generic reagents for molecular biology (Sigma-Aldrich-Milan, Italy) and specific "primers" (Chemicon International, Temecula, CA, USA) for the various mRNA related to the molecules under study.

Analysis of genes expressed on cells that codify for adhesion proteins and for proteins that control cellular movement:

I. urochinase Plasminogen Activator (uPA), urichinase Plasminogen Activator Receptor (uPA), Plasminogen Activator Inhibitor (PAI);
II. matrix-metal proteases 2 and 9 (MMP2 and MMP9);
III. inhibitors of metal proteases (TIMP-1 and TIMP2).

Protocol

The mRNA expression of genes under examination has been determined with:

1) Extraction of RNA
2) RT-PCR
3) Analysis of data

1) Extraction of RNA

Biopsis where centrifuged in Eppendorf test tubes. 500 micro-liters of Trizol are added to every test tube and is left to rest for 15 minutes at room temperature. Then 100 ml of chloroform are added and the test tube is shaken vigorously by hand for 15 seconds and then left to rest at room temperature for 2 to 3 minutes. Then it is centrifuged at 4°C for 15 minutes at 12,000 RPM. The liquid part is transferred to another test tube taking care not to take any of the underlying material which is to be thrown away. 250 microliters of isopropanol are added. The test tube is shaken and then incubated in ice for 10 minutes. It is then centrifuged for 10 minutes at 4°C at 12,000 RPM. The test tubes are then put in ice and the above liquid is then removed. To the remaining pellet is added 0.5cc of ethanol at 75%. Again it is centrifuged at 12,000 RPM for 5 minutes at 4°C. The substance above is removed and the residual ethanol is evaporated putting the test tube containing the pellet either in a warm room or under a lamp for about 30 minutes. When the ethanol has dried 40 microliters of distilled water are added to the RNA pellet (better still if Nucleas free water is used) and the sample is re-suspended and conserved in a freezer at -20°C. After the extraction of the RNA, the spectrophotometer is used to evaluate the quantity and purity. The ratio of OD260/OD280 close to 2 indicates that RNA has a good degree of purity.

2) Real Time PCR

The PCR is based on the use of an enzyme, the Taq polymerases that catalysis the reaction of amplification in vitro of a particular sequence of DNA starting from a fraction of nucleic acid which is utilized as mold. The Real Time PCR may be obtained with use of intercalating dyes (ex: SYBR green) which connect themselves in a specific manner to the whole DNA or with hybridization probes specific for the amplified fragment of the transgene under study marked with fluorescent molecules as in our case.

B) Analisys of Histology and Immunohistochemistry

The tissue collected has been submitted to conventional histological investigations (hematoxylin-eosin) to evaluate the characteristics of the newly formed mucosa above the head of the cover screws and the possible presence of a inflammatory infiltrate.

Immune histochemical studies have been carried out to evaluate the expression:

I. of the receptor and of uPA in the stroma and in the perivascular infiltrate;
II. of nuclear antigens of proliferation (Ki67) on epithelial cells.

Protocol

Every fragment of newly formed mucosa removed from above cover screws has been quickly fixed by immersion in 4% formaldehyde in PBS (phosphate-buffered saline) 0.2 M pH 7.4, for 8 hours at room temperature.

Fragments have been washed again in PBS and closed in paraffin and sectioned with conventional microtome in slices of 6µ of width.

After eliminating paraffin some sections have been colored with hematoxylin-eosin for conventional histology.

Consecutive sections have been deparaffinated, submitted to treatment to reacquire antigenicity (target retrieval solution, Dako, Milan, Italy) and subsequently immune-marked with primary antibodies specific for the molecules under study. In particular rat monoclonal antibodies (Dako) have been used: anti-uPAR (dilution used 1:30) e anti-Ki67 (dilution used 1:100).

The identification of specific immunoreactions sites has been obtained using suitable secondary antibiodies and the immunoreaction has been evidenced with avidina-preossidases (Dako), using a solution of 3-3'diaminobenzidina and H_2O_2 as chromogenic system. The nuclei have been

colored with hematoxylin.
The material so obtained has been observed and photographed with a Nikon optic microscope equipped with digital fotocamera.

Results

Clinical Phase

2 male and 2 female patients have been enrolled. Each patient has received 4 implants inserted at crestal level for a total of 16 implants. 16 cover screws have been inserted of which 8 laser treated and 8 sandblasted (Table 1)

Table 1. Distribution of patients by gender, type of edentoulism and rehabilitation. (F=female, M=male)

	GENDER	TOTAL INFERIOR EDENTULISM	BILAT. INF. KENNEDY 3° CLASS	BAR RETAINED OVERDENTURE ON 4 INPLANTS	BILATERAL FIXED BRIDGE OF 3 ELEMENTS ON IMPLANT
Patient 1	F	X		X	
Patient 2	M	X		X	
Patient 3	F		X		X
Patient 4	M		X		X
Total		2	2	2	2

Gender, class of endentulism and type of final prosthesic rehabilitation have been distributed as follows.
2 patients, a male and a female, with total lower edentulism have received 4 implants each. The programmed prosthesic rehabilitation is bar-retained overdenture.
2 laser treated cover screws have been inserted in the quadrant and 2 sand-blasted cover screws in the fourth quadrant.
Two patients, one male and one female, with a bilateral third class of Kennedy, have received 4 implants each. The programmed prosthesic rehabilitation is a bridge of three elements in the posterior and inferior region. Two laser treated cover screws have been inserted in the third quadrant and two sandblasted cover screws have been inserted in the fourth quadrant. 6 tissue samples have been taken for each patient at the established times for a total of 24 samples (Table 2). Each sample has been subdivided by area of migration in 2 speciments, one to be sent for studies of molecular biology and one for studies of histology and immunohystochemistry (Fig 1).

Table 2. Distribution of implants, of cover screws and of samples collected

	2 IMPLANTS PER QUADRANT	LASER-TREATED COVER SCREWS 3RD QUADRANT	SANDBLASTED COVER SCREWS 4TH QUADRANT	T-0 CONTROL SAMPLE 3RD + 4TH QUAD.	T-1 SAMPLE AT 7 DAYS SAND + LASER	T-2 SAMPLE AT 40 DAYS SAND + LASER
Patient 1	4	2	2	2	2	2
Patient 2	4	2	2	2	2	2
Patient 3	4	2	2	2	2	2
Patient 4	4	2	2	2	2	2
Total	16	8	8	8	8	8

Laboratory phase

A) Studies of molecular biology

The genes have been analyzed from samples taken from healthy gum tissue (control) and from samples of tissue at the contact of two different implant surfaces (sandblasted and laser treated). These are genes that codify for proteins involved in the cellular movement and in particular the urokinasic Plasminogen Activator (uPA), uPA Receptor (uPAR), Plasminogen Activator Inhibitor (PAI - 1), metal proteases 2 and 9 (MMP2 and MMP9) and inhibitors of metal proteases (TIMP-1 and TIMP2).

Samples obtained from 4 patients have been sent to be studied, on each one of which two laser treated and two sandblasted cover screws have been implanted respectively.
Only for three patients it has been possible to carry out molecular biology analysis on the genes examined. (insufficient material in the fourth patient).
The first sample has been taken after 7 days and a second sample some time later (30 to 40 days).
Results obtained refer only to samples taken after 7 days (Figures 2 and 3).
As regards the second sampling some time later it has not been found any significant difference between the two types of screws.
This is probably due to the fact that such sampling has been carried out at different times in the different patients and in any case after too long time when healing has been completed and was stabilized and therefore the cells "did not need to move anymore".
On all samples a major expression of the uPA gene has been found as well of its receptor uPAR and of the genes MMP2, MMP9 on laser treated surface. Conversely a reduction has been observed on the expression of the inhibitor PAI1 on the laser treated versus the sandblasted one. Inhibitors of MMP are more evidently expressed in tissues grown in contact with the laser treated surface. Nevertheless it is necessary to underline that this phenomena are not specific but they are present also in other cellular processes (Figures 2 and 3).

Figure 2. Expression of genes that codify for urokinase plasminogen activator (uPA), uPA receptor (uPAR) and uPA inhibitor (PAI-1). In this figure results are given for patients 1 and 3

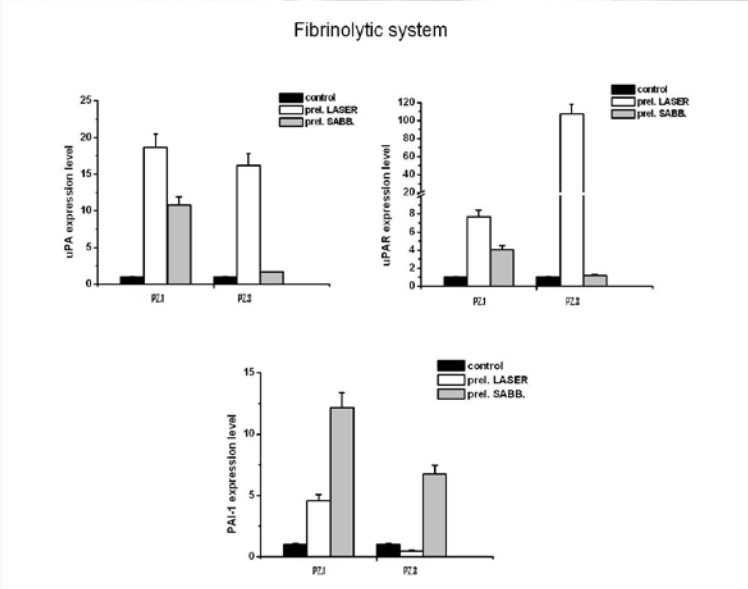
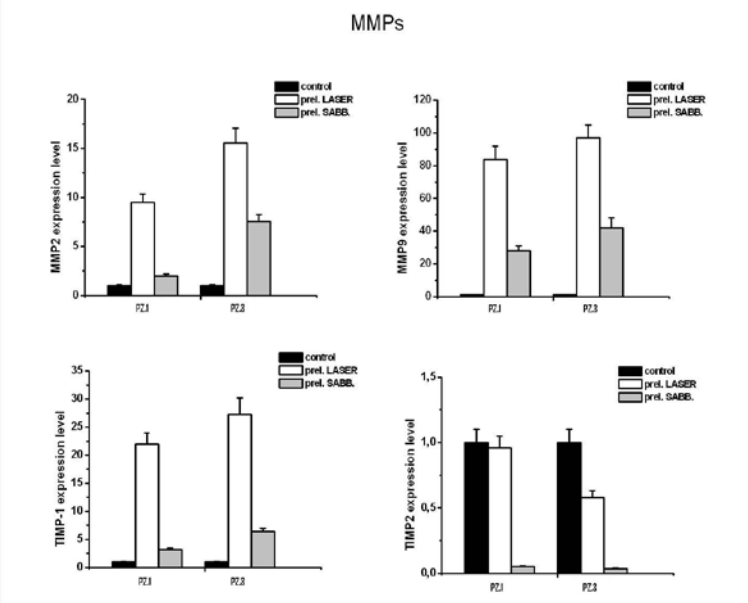


Figure 3. Expression of genes that codify for matrix metalloproteasis 1 and 9 (MMP 2 and MMP 9) and tissue inhibitors of the MMPs (TIMP-1 e TIMP-2). In this figure results are given for patients 1 and 3



B) Histology and Immunohistochemistry

As specified in "methods" the sections of tissue obtained from biopsies fixed in 4% formaldehyde and enclosed in paraffin have been immune-marked for the detection of the antigen of proliferation Ki67 (Dako, 1:100) and for the receptor uPAR (Dako, 1:30).

Morphometric analysis has been carried out evaluating:

I. for the stromal cells expressing uPAR the number of cells per microscopic field ()area of 83.750 mm^2)
II. for the proliferating cells the ratio (Ki67+cells) / (total cells) counted on the basal layer of the gum epithelium

For static reasons, for every experimental point, three randomly chosen microscope fields have been counted.

I. Immunohistochemical studies for the identification of the uPA receptor (uPAR) in the stroma and the perivascular infiltrate.

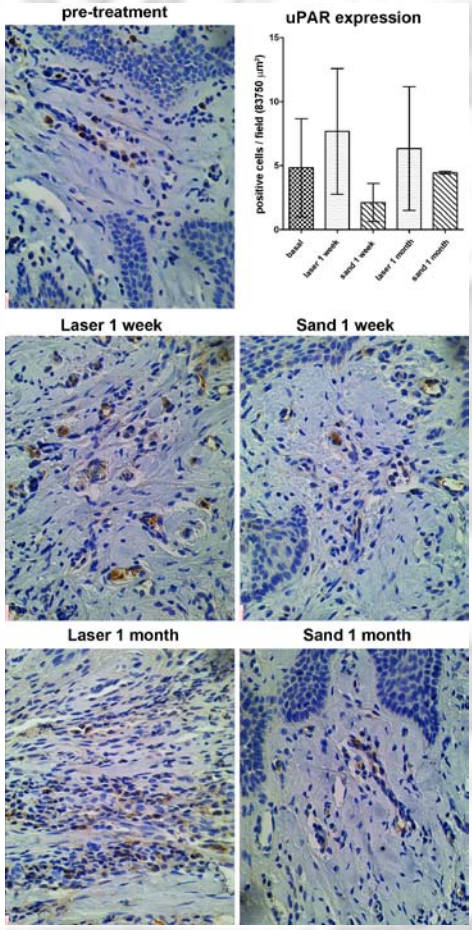
In the samples of normal mucosa, before the treatment, uPAR + cells have been found in the stroma and in the perivascular infiltrate.

After one week and one month from the positioning of the implant, uPAR + cells expected to fix and activate plasminogen favoring adesion and cellular mobility processes were significantly higher in samples of mucosa obtained from above the laser treated cover screw as compared with those obtained above the sandblasted cover screws, this is in full agreement with the data obtained from tests of molecular biology (Figure 4).

The wide variability among the three subjects analyzed makes differences statistically not significant.

Values given in figure 4 are related to patient number 4 at one week.

Figure 4. Detection of uPA receptor (uPAR), counting of number of uPAR+ cells per microscope field after 1 week and 1 month. (before treatment, 1 week, 1 month)



II. Immunohistochemical studies for the identification of nuclear antigens of proliferation (KI67) on connective and epithelial cells.

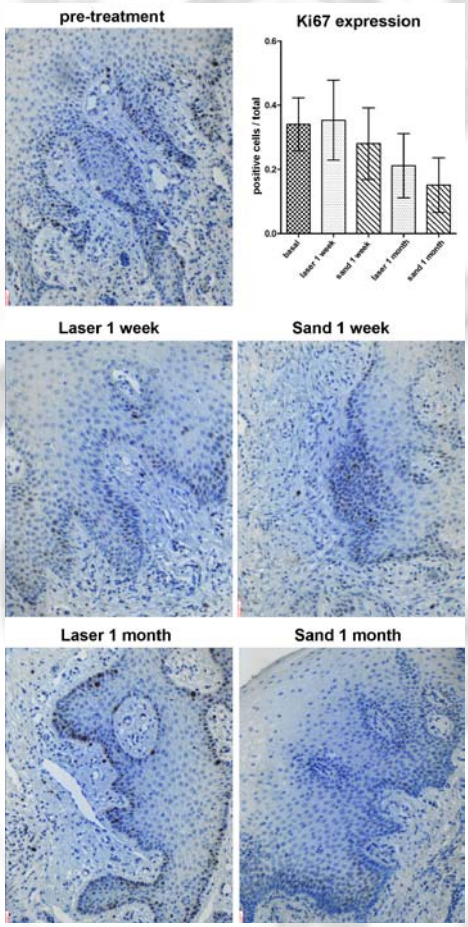
In samples of normal mucosa before the treatment Ki67+ cells have been found in the basal layer of the mucosa as usually happens in this type of tissue.

After one week and one month from the implant positioning, the number of Ki67+ epithelial cells was slightly higher in samples of mucosa taken from above the laser treated cover screw as related to those taken from above sandblasted cover screw (Fig 5).

The large variability among the three subjects analyzed makes the differences statistically not significant.

The values given in figure 5 refer to patient number 3 at 1 week.

Figure 5. Detection of Ki67 antigene: counting of number of Ki67+ cells per microscope field after 1 week and 1 month. (before treatment, 1 week, 1 month)



Conclusions

Results obtained from studies of molecular biology after the seventh day sampling, seem to suggest a greater expression of genes involved in the activation of proteolytic mechanisms of degradation of the collagenic and non-collagenic components of extracellular matrix in the tissue grown in contact with the laser treated surfaces as compared with those grown in contact with the sandblasted surfaces.

The results of morphometric investigations on immunohistochemistry confirm those of the molecular biology. However also in this case the limitation of the analyzed sample doesn't reach a statistical significance. In fact in tissues healed in contact with laser treated lid screws as compared with those sandblasted, it is possible to find a larger number of cells that express uPAR that are able to fix and activate plasminogen favoring processes of adhesion and cellular mobility as well as a larger number of basal epithelium cells in proliferation.

These preliminary data, in spite of the limited number of patients and therefore of the samples

taken, deserve confirmation by further studies with a larger sample of patients. Nevertheless, these data are to be kept in mind in the analysis of induction caused by different surfaces in titanium on the healing mechanisms of soft peri-implantar tissues.

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