Biochemical and histological evaluation of human synovial-like membrane around failed total hip replacement prostheses during in vitro mechanical loading

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The biochemical role of the synovial-like membrane formed at the interface of eight aseptic failed total hip prosthesis has been investigated during in vitro mechanical loading. The study was carried out on four membranes from cemented prosthesis and four titanium alloy uncemented ones. Intermittent positive pressure leading to 20% deformation of the membrane (100 g/cm²) was applied to the membrane fragments in cycles (300 cycles/15 min) repeated three times at thirty minutes intervals in which interleukin-6 (IL6), prostaglandin-E2 (PGE2) and interleukin-1β (IL1β) levels were quantified both in culture media and in tissue extracts. Histological, morphometrical and immunohistochemical studies were also carried out on the same membranes.

Mechanical stress evidenced an increase in the release of the examined cytokines both in cemented and uncemented prosthesis tissues; particularly evident was IL6 trend of increase from cemented prosthesis and IL1β result from uncemented ones. Histomorphological and immunohistochemical data revealed no differences between membranes obtained from cemented and uncemented prosthesis as to cell proliferation, fibrosis, macrophages lymphocytes B and T population, vessels and nervous fibers. The results indicate that mechanical stress plays a fundamental role in increasing membrane production and release of cytokins known as bone-resorbing agents. Furthermore, the histologic finding of synovial-like membrane with the same histomorphological and immunohistochemical findings but with different biochemical response to mechanical stimulation, suggests that cells involved in the production and release of the considered mediators might have different strain behavior by different development conditions (previous contact with PMMA).
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1. Introduction
It is estimated that between 300,000 and 400,000 total hip replacements are inserted each year worldwide. In initial experiences poor clinical results were most often related to mechanical failure such as fracture of the prosthetic components or postoperative infections. Modifications of the surgical techniques and refinements in the prosthetic materials used have drastically reduced the incidence of these complications [1]. Currently, the most common cause of clinical failure is aseptic loosening of the components at the implant site [2]. Long term studies have shown a radiographically detectable zone of radiolucency which developed at the material-tissue interface in correspondence of a fibrous membrane [3]. Although numerous descriptions of the histological and histochemical features of this membrane have been published in literature [4], the origin of this tissue, the biological signals responsible for its formation and its role in progressive bone resorption associated with loosening are still poorly understood.

The nature of the membrane and its effect on the surrounding bone are of critical importance for the implications in bone resorption. Biochemical studies performed on these so-called synovial-like membranes retrieved during implant revision procedures have revealed the presence of molecules known as bone-

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resorbing agents such as interleukin-6 (IL-6), interleukin-1β (IL-1β), prostaglandin-E2 (PGE2), tumor necrosis factor-alpha (TNFα), transforming growth factor-beta (TGFβ), which may play an important role in the development of aseptic loosening and osteolysis. In fact, membranes that surrounded components with radiographic evidence of diffuse or localized periosteal bone loss released significantly more collagenase, IL-1, IL-6 and TNFα than membranes from components without bone loss, suggesting a macrophage activation role and the release of mediators of bone resorption in the membranes surrounding failed implants [5].

The variables affecting the production of these factors have been widely studied. Emphasis has been placed on the presence, in these membranes, of metal and polymeric wear particles released by the prosthetic components and in vitro studies have shown that the release of some bone-resorbing agents is influenced by size, concentration, composition, surface area and volume of biomaterial particle debris [6–10].

While it has been clearly demonstrated that biomaterial particle release can induce perimplantar bone resorption, it is still debatable whether this is the only factor inducing implant loosening; the presence of other factors is suggested by the clinical observation that identical implants have a different duration in time, and is further supported by our personal histologic finding of synovial membrane with a very small amount of particles retrieved from patients with radiographical signs of severe loosening.

Recently, attention has been focused on the possible role of mechanical forces in bone remodelling [11]. Mechanical stress was shown to evoke biomechanical and structural responses causing perturbation of membrane phospholipids, leading to the release of arachidonic acid and the subsequent synthesis of PGs [12]; also the phospho-inositol pathway was activated by mechanical stress [13]. Human periodontal ligament fibroblasts were shown to produce PGE and IL-1beta in response to intermittent positive and negative pressures [11,13].

In order to extend our knowledge about the biochemical role of the membrane formed at the interface of aseptic failed hip cemented and uncemented prostheses, we studied in vitro, the effects of cyclic loading on the membrane release of bone-associated cytokines (IL6, IL1β and PGE2) as representative bone-resorbing agents [14–16].

2. Materials and methods
2.1. Collection of tissue specimens
Tissue specimens were obtained at the time of surgery from eight patients (male and female 68 ± 6 years old) undergoing acetabular component revision for aseptic failure. The eight membranes were retrieved from four cemented prosthesis and four titanium alloy uncemented prosthesis. The uncemented acetabular components revised were Ti6Al6Va threaded cups (SCL, Lima, Italy) coupled with SCL stems; the cemented cups were standard UHMWPE cups (Charnley, De-Puy Inc., Warsaw, USA), coupled with cemented Charnley stems. All patients in this study had radiographic and clinical evidence of prosthetic loosening and had discontinued any antiinflammatory medication ten days before revision surgery. Patients with rheumatoid arthritis or malignancy or the presence of infection were excluded by appropriate bacteriologic and histologic evaluation of tissue specimens. The tissue samples were taken from the zone II of the radiographic classification of DeLee-Charnley, corresponding to the back of the component. No tissue was taken from areas that could have been considered to show aggressive lysis. Matching samples of tissue were prepared for biochemical, histologic and immunohistochemical analysis.

2.2. Organ culture
Organ cultures were carried out as described by Goldring et al. [2]. Briefly, tissue from the lining membrane from the implant interface was cut into two small fragments (5–10 mm) weighed and placed in 30 ml organ culture chamber (Ugo Basile). The fragments were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with 100 units of penicillin and 100 μg of streptomycin/ml at a temperature of 37 °C in a moist atmosphere of 5% carbon dioxide and 95% air. One of the two tissue fragments was used for the application of mechanical forces and the other was kept under no stress conditions.

2.3. Application of mechanical forces
A force transducer (Instron 5564, Instron Co., Canton, Mass.) connected to a software Program (Merlin, “A” version, 1993) which could be operated either continuously or intermittently by setting load, time, cycle and strain, was used for the application of mechanical force to tissue cultures. Membrane fragments located in the organ culture chamber were left to rest for one hour before beginning the test. Then membranes put on a stiff disk of 15 mm diameter were subjected to intermittent pressure using a polyethylene punch of 10 mm diameter, leading to 20% deformation of the membrane as revealed by the dimension of the sample. Then, intermittent positive pressure of 100 g/cm² was applied to the membrane fragments in cycles (300 cycles/15 min) repeated three times at 30 min intervals.

These parameters have been used after preliminary observations for the maximum deformation not inducing to cell membrane damage by quantification of LDH release and the theoric engagement during normal walking.

2.4. Biochemical assay
Interleukin 6, Interleukin 1β and Prostaglandin E2 were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kit from Amersham (Amersham Life Science, Milan, Italy). Biochemical assays in culture media were done on drawings at four different times: a first drawing, used as an initial control, was made after the membrane had been left to rest for one hour in the culture organ chamber, the second drawing was made at the end of the first cycle compression, the third at the end of the second cycle
compression and the last at the end of the mechanical stimulation test. Lactate dehydrogenase activity was measured using the principle of oxidation of an equimolar amount of NADH to NAD⁺ during the reduction of pyruvate to lactate. The oxidation of NADH results in a decrease in the absorbance at 340 nm which is directly proportional to lactate dehydrogenase activity in the sample [17].

The data thus obtained were statistically examined using Mann–Whitney U non-parametric test; Friedman's test was used when comparing data at the beginning and end of mechanical stress experiment.

2.5. Histology and morphometry
Periprosthetic membrane samples were removed from the implants and fixed in 10% phosphate buffered formaldehyde. After a macroscopic observation of the pseudomembrane, capsular tissue samples were dehydrated in increasing ethanol concentration, cleared in xylol and embedded in paraffin for each subject. Serial 4 μm thick sections from the same tissue blocks were cut and used for histology, and immunostaining.

Haematoxylin-eosin stained sections were analyzed with a Leitz light microscope equipped with a digital camera. Quantitative results and scores were given as the average of the analyses performed on ten fields for each sample. Analyses were performed at medium-powered magnification (250 × ) for the evaluation of the overall cellularity, fibrosis and amount of multinucleated giant cells, and high-powered magnification (500 × ) was used to evaluate the polyethylene particles.

A semi-automatic image-analysis program was used to assess the overall cellularity per field and the amount of particles (GIPS 12D, Gade Data). The image analyzer was calibrated at a chosen magnification level of 100 × and in a range of grey scale level sensitive to nucleus and/or polyethylene particle resolution. As the computer system is able to count the number of objects that have the same intensity in the grey scale, nuclei or particles were identified according to the grey scale gradient of the structures when compared to other parts of the specimens.

Two different observers carried out a blind scoring of the fibrosis, defined as interstitial mature collagen deposition with scarcity of fibroblasts: a score of 1 was given in the absence or minimal presence of fibrosis, 2 when moderate, 3 when extensive; the overall score was given as the average score from ten fields from each sample [18]. According to the classification of Mirra, the multinucleated giant cells were scored as 1 (1–2 cells per field), 2 (3–8 cells) and 3 (9 cells or more per field) [19]. The samples were scored, for particles, according the classification of Jiranek [18], as 1 (less than 10 particles per field), 2 (10–19 particles), 3 (20–49 particles) and 4 (50 particles or more per field).

2.6. Immunohistochemistry
Immunohistochemical staining was carried out using 4 μm thick sections mounted on 0.01% w/v polylysine-coated slides, dried overnight at 45 °C. The sections were then placed in a glass beaker containing 10 mM citrate buffer pH 6.0 and subjected to microwave irradiation at 80 W for 15 mins and at 40 W for a further 5 min.

To detect reactive macrophages and histiocytes, the sections were then rinsed in phosphate buffered saline and incubated with CD68 monoclonal mouse anti-human macrophage antibodies, clone KP1, CD3 monoclonal mouse anti-human T cell antibodies, clone UCHL1 against reactive T cells; CD 20 monoclonal mouse anti-human B cell antibodies, clone L26 for reactive B cells (all from Dako, Denmark manufacture). In order to detect vessel endothelia, CD34 monoclonal mouse anti-human endothelial cell antibodies (clone JC/70A Dako), which reacts with a glycoprotein of MW 100kD in endothelial cells, were used. The MIB-1 immunostaining was carried out on sections incubated in 1:100 diluted monoclonal antibody solution (Immunotech, Marseille, France) for 2 h at room temperature. Anti-S-100 polyclonal antibody (Immunon, Pittsburgh, USA) was used to evidence nerves and anti-pan-actine monoclonal antibody clone, A4 (Dako) was used to evidence smooth muscle cells. The labeled Streptavidin Biotin (LSAB) method (Dakopatts, Glostrup, DK) was employed for immunostaining using diaminobenzidine as the chromogen.

3. Results
3.1. Biochemical results
Mechanical forces applied to the tissue evidenced no lactate dehydrogenase increase during the experiment (Fig. 1a) for membrane from cemented and cementless prostheses. This data showed the presence of no direct effect of intermittent positive pressure on tissue cell membranes.

Interleukin-6 quantification in culture media evidenced a higher, with no statistical relevance, initial control value in membranes explanted from cemented prosthesis 4.62 ± 1.16 pg/mg respect to uncemented ones 1.6 ± 1.25 pg/mg. During mechanical stimulation, an increase of IL-6 levels was noted and was particularly evident at the end of the experiment with a significantly higher value for cemented prosthesis pseudomembrane 59.17 ± 45.08 pg/mg compared to 6.24 ± 3.88 pg/mg for uncemented ones (p < 0.05 Mann–Whitney U test) (Fig. 1b).

PGE2 quantification in culture media showed a statistically higher initial value in membranes obtained from cemented prosthesis 1.13 ± 0.76 ng/mg compared to uncemented ones 0.08 ± 0.06 ng/mg (p < 0.05 Mann–Whitney U test) such difference consists in a 1.5-fold increase during mechanical stimulation, as PGE2 culture media value at the end of the experiment is 2.9 ± 2.9 ng/mg for cemented and 0.2 ± 0.17 ng/mg for uncemented prosthesis (Fig. 1c).

IL1β results on culture media evidenced a significantly higher data 0.67 ± 0.41 pg/mg in membranes obtained from cemented prosthesis compared to uncemented ones 0.06 ± 0.04 pg/mg before starting mechanical stimulation (Fig. 1d). During mechanical stress, no increase in IL1β value is observed in membrane culture media from cemented prosthesis, whereas membranes from uncemented ones showed a 5.6-fold increased release so no differences in IL1β level resulted between cemented
and uncemented membranes at the end of the experiment.

3.2. Histologic and morphometric results
There was no significant difference between membranes removed from cemented or uncemented prosthesis, at histologic examination. Our findings were very similar to those of Goldring [2] and Lennox [19].

The membrane at cement or titanium interface presented a synovial-like layer formed of teardrop-like/globular cell infiltrates. Below this interface, mononuclear cells and rare foreign-body giant cells around polymeric debris, were observed. Most of the bone interface layer was formed of fibrous tissue with rare osteoclasts. No difference was found between membranes retrieved after contact with cement or titanium alloy materials, at morphometric analysis.

A moderate fibrosis was observed with a mean cell number of 40 cells/100 μm², 3–8 multinucleated giant cells per field and 10–20 birefringent particles per field (Table I).

3.3. Immunohistochemical results
The synovial-like membranes removed from cemented and uncemented loosened prosthesis, showed no significant differences and both had the same macrophage population. In all cases a positive CD68 population was evidenced (Fig. 2a) together with a few B (CD20) and T (CD3) lymphocytes. CD34 showed the presence of numerous vessels (Fig. 2b) and of a few S100 positive nerves.

A rather high MIB1 positive cell proliferation was also evidenced in the pseudomembranes (Fig. 2c), with fibroblast and smooth muscular cell activation (pan-active positive) as shown in Fig. 2d.

4. Discussion and conclusions
Over the last 15 years great emphasis has been set on the role of the synovial-like membrane surrounding loose prosthetic components in the pathogenesis of the implant loosening. In this study, we have shown that membranes with the same histomorphological and immunohistochemical characteristics retrieved from aseptic failed cemented and uncemented total hip prosthesis evidenced a different release and production of IL6, PGE2 and IL1β. It has been clearly demonstrated that particles released by the prosthetic components may act as foreign bodies inducing an inflammatory response with subsequent bone osteolysis [18].

These particles seem to have stimulating effect on the production of cytokines released by the macrophages in
the membrane. All these data led Maguire and coworkers [16] to develop a hypothesis of loosening that is centered on the foreign body giant cell reaction; if this hypothesis alone can be considered sufficient to explain implant loosening in cases with radiographical appearance of severe cavitory bone defects, it does not seem to be able to explain the cases of early loosening with only few radiographical signs of failed osteointegration or cases shown here with the same small amount of particulate debris, the same entity of cell proliferation, the same population of macrophage lymphocytes and vessels, the same fibrosis and different biochemical data.

A second important part of our study evidenced that mechanical compression applied to these retrieved hip pseudomembranes was able to induce an increase in the release of the examined cytokines that were demonstrated to be able to determine osteoclast differentiation and ultimately bone resorption [13, 14]. These results are very close to the findings of Saito as to the PGE2 production by human fibroblast having undergone mechanical compression [13].

Of particular interest was that the pseudomembranes studied responded in a different way to mechanical stimulation. Membranes from cemented prosthesis, that have the highest initial levels of the considered three factors responded to mechanical stress with an increase only in IL6 and PGE2 release from culture media data. Trends of increased release, at short time intervals, as revealed by culture media data was equal for the two kinds of membranes for PGE2, while uncemented ones

### Table I Morphometric analysis results

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Fibrosis is defined as interstitial mature collagen deposition with scarcity of fibroblasts; a score of 1 was given in absence or minimal fibrosis, 2 when moderate, 3 when extensive; multinucleated giant cells were scored as 1 (1–2 cells per field), 2 (3–8 cells), 3 (9 or more cells per field); particles are scored as 1 (less than 10 particles per field), 2 (10–19 particles), 3 (20–49 particles) and 4 (50 particles or more).

Figure 2 (a) The immunohistochemical staining with CD68 shows a large positive macrophage population (×200). Example of a membrane obtained from cemented prosthesis in which the levels of proinflammatory agents were not high. (b) The CD34 staining shows a large number of positive vessel endothelia (×200). (c) The MIB1 staining evidences a rather high cell proliferation (×200). (d) The pan-actin staining shows activated fibroblasts and muscular cells (×200).
responded to mechanical stress with a higher IL1β release and cemented ones with higher IL6 release.

These differences in biochemical response to mechanical stimulation suggest that, like osteoblastic response to mechanical strain [20], macrophages and fibroblasts involved in the release of the considered mediators have a different mechanical loading secretive response in different conditions of development increasing the release of different cytokines. However, bone resorption induced by proinflammatory mediators is functionally neutralized by anti-inflammatory factors, and bone resorption is determined by the overall balance of these factors [21].

References

Received 24 May and accepted 25 October 2000