Cell proliferation rates and fibronectin arrangement as parameters for biocompatibility evaluation of dental metal alloys in vitro

Vittorio Grill§, Maria A. Sandrucci§, Roberto Di Lenarda‡, Elettra Dorigo‡, Paola Narducci§, Alberto M. Martelli§ and Renato Bareggi§

§ Department of Human Morphology, University of Trieste, Via Manzoni 16, 1-34138 Trieste, Italy
‡ Institute of Odontology and Stomatology, University of Trieste, Via Stuparich 1, 1-34125 Trieste, Italy

(Received 9 August 1999 and accepted 19 January 2000)

Abstract: A short-term (72-96 hours) biocompatibility evaluation in vitro of four single phase dental metal alloys was conducted by determining cell proliferation rates correlated to the organization of the extracellular matrix protein fibronectin in human fibroblast cultures. Immunocytochemical methods were performed to detect both cell proliferation rates by 5-bromodeoxyuridine (BrdU) incorporation, and fibronectin arrangement, i.e., diffuse in the extracellular matrix, organized in fibrils or in focal adhesions. We showed that cell proliferation rates were related to fibronectin expression. In particular, a higher percentage of cells in the S-phase were related to a predominance of fibronectin organized both in fibrils and in focal adhesions. The alloy with the highest Au content seemed the most biocompatible among those tested, since it behaved in a very similar manner to the controls. On the contrary, fibroblasts exposed to the alloy with the highest percentage of Ag had the most different behavior as compared to the controls. We can assume that a correlation exists between fibronectin organization and the percentage of BrdU-positive cells and that these parameters are varying with the different metal composition of the alloys. The observation of fibronectin arrangement together with cell proliferation rates could be considered a useful tool to determine the biocompatibility of these biomaterials. (J. Oral Sci. 42, 1-7, 2000)

Key words: dental alloys; cell proliferation; fibronectin; biocompatibility.

Introduction

In the recent years, the influence of dental metal alloys on cell viability and proliferation has been widely studied to show their biocompatibility in vitro (1-4). Although in clinical practice it may be useful to test the behavior of dental metal alloys in vivo, many factors could influence these experimental observations, since local inflammatory or other adverse reactions could frequently occur (5). Studies in vitro can give more significant results, since they allow evaluation of the behavior of a single cell line in regards to cell viability and proliferation, as well as cell adhesion to the substrate (6-8). Many investigations on biocompatible materials have recently focused on the interaction between cells and the metal cations released from dental metal alloys under certain conditions (3,8-10). The so named "noble elements" Au and Pd cannot be found in significant amounts in the culture media, whereas Ag, Cu and Ga are always detectable (9,10). A correlation between the atomic weight percentage (at%) of Ag and Ag⁺ released in the medium, dependent on a second-order polynomial relationship, when the at% of Ag is higher than 10% in single phase alloys has been reported (2).

Cell proliferation rates can be determined by calculating

Correspondence to Dr. Vittorio Grill, Department of Human Morphology, Via Manzoni 16, I-34138 Trieste, Italy
the percentage of cells in the S-phase of the cycle and this is much easier to perform by 5-bromodeoxyuridine (BrdU) immunocytochemistry than by the incorporation of H-thymidine (11,12). Another parameter that should be considered in the biocompatibility evaluation of biomaterials is cell adhesion to substrates, since it influences cell proliferation (13, 14). Fibronectin is one of the molecules involved in cell adhesion and consists of two different polypeptide chains with a disulphide bond at the carboxyl end (15). Fibronectin is organized into fibrils in the extracellular matrix and this is a multi-step process (16). Fibronectin appears to be also arranged in focal adhesions, that play an important role in cell locomotion and in cell adhesion. In fact, to grow in culture, cells must adhere to substrates by means of focal adhesions in order to develop microfilament bundles to anchor and flatten the cell body against the substrate (17,18).

In regards to the cell models employed, primary culture systems, i.e. gingival fibroblasts, do not always exhibit reproducible results, whereas stabilized cell lines are much easier to be maintained (14).

On these bases, the aim of the present work was to determine the influence of four single phase dental metal alloys with different metal compositions on the Flow 2002 human fibroblast stabilized line. We evaluated cell proliferation rates, by determining the percentage of cells in the S-phase by BrdU immunocytochemistry, and the relationship between cell proliferation and fibronectin arrangement, after 72 and 96 h culture times.

**Materials and Methods**

**Dental metal alloys**

Blocks of four single phase dental casting alloys, weighing from 1.24 to 1.90 g and with dimensions of 11 mm x 8 mm x 0.7 mm were tested as purchased by the manufacturer, after rinsing in pyrogenic-free water and sterilization with ultraviolet rays for 24 h. The composition both in weight percentage (wt%) and atomic weight percentage (at%) of each alloy is indicated in Table 1.

**Cell culture and treatments**

Human fibroblasts (line FLOW 2002) were grown in 75 cm² flasks in Dulbecco’s Modified Eagle Medium (MEM; Sigma, St. Louis, Mo, U.S.A.) supplemented with 10% fetal calf serum (FCS, Sigma), 100 units/ml penicillin and 100 (g/ml streptomycin sulfate, at 37°C in a fully humidified air atmosphere containing 5% CO₂.

For the cell proliferation assay, 10⁴ cells were added to 5 ml of culture medium and grown for specified times (72 and 96 h) - without renewing the medium - at approximately 40% confluence, in 150-mm Petri dishes, in which sterile coverslips and one metal alloy block were immersed. As controls, a further series of cultures was performed in the same conditions in the absence of alloys. In each test, duplicate samples were run and each experiment was repeated five times, for a total of ten replications.

At each time period, replicate cultures growing adhered to coverslips were treated with 10⁻⁶ M BrdU (Sigma) for 20 min at 37°C in the same culture medium. After washing in PBS, coverslips were fixed using freshly made 4% paraformaldehyde in PBS (pH 7.3) for 30 min at room temperature, then quickly washed in PBS, dehydrated in 4N HCl for 30 min, dehydrated in graded ethanol (70%, 90%, 100%) and air dried. The samples were then further processed.

For the fibronectin arrangement assay, fibroblasts were grown for the same times as above. Coverslips were then fixed in 4% paraformaldehyde in PBS (pH 7.3) for 30 min and subsequently underwent immunocytochemical procedures.

**BrdU immunocytochemistry**

After treatment with BrdU, coverslips were incubated with a monoclonal mouse anti BrdU antibody (Sigma) diluted 1:20 in 2% bovine serum albumin (BSA) -3%-normal goat serum (NGS) in PBS, for 3 h at 37°C. A prior permeabilization step was not required as the denaturating HCl effectively permeabilizes the cells. After three washes in PBS, antibody binding was detected by incubating slides for 30 min at 37°C with fluorescein isothiocyanate (FITC) -conjugated anti mouse IgG (Sigma) used at 1:50 dilution in 2% BSA-3% NGS in PBS. Slides were washed three times in PBS, dehydrated in graded ethanol (70%, 90%, 100%) and then mounted using glycerol containing 2.3% 1,4-diazobicyclo (2.2.2.) octane (BDH, Poole, England) - 0.02% NaN₃ - 20 mM Tris-HCl pH8 (glycerol-DABCO) to delay fading. Finally, a Zeiss Axioptot microscope was used to examine samples under fluorescence and phase-contrast conditions.

10³ cells from randomly selected fields were counted in each sample. Cell counts were always made by the same
operator. The BrdU-positive cells were expressed as a percentage of the total number of nuclei and the average values (n = 5) and standard deviations (SD) were calculated. The difference between the mean percentage of BrdU-positive cells in each sample and that of the control at the same culture time was evaluated with the Student’s paired t-test, taking p ≤ 0.05 as statistically significant.

Fibronectin immunocytochemistry

Cells grown on coverslips were fixed as above, washed three times with PBS and incubated for 1 h at 37°C in 4% BSA-5% NGS in PBS to block non-specific binding.

Coverslips were then incubated for 3 h at 37°C with a rabbit anti-human plasma fibronectin serum (Sigma) diluted 1:100 in 4% BSA-5% NGS in PBS. After three washes with PBS, the samples were reacted for 1 h at 37°C with a 1:50 dilution of FITC-conjugated anti rabbit IgG in 4% BSA-5% NGS in PBS. After three washes with PBS and a subsequent dehydration with graded ethanol (70%, 90%, 100%), they were mounted in glycerol-DABCO. Finally, they were observed using a Zeiss Axiophot microscope equipped for epifluorescence. Photographs were taken on Kodak TMAX 100 film.

Results

BrdU immunocytochemistry and statistical evaluation of the percentage of BrdU-positive cells.

The percentages of BrdU-positive cells are summarized in Table 2. At 72 h, samples grown in the presence of metal alloys showed a percentage of BrdU-positive cells ranging from 22.97 ± 3.79 (L/15I) to 31.49 ± 4.69 (L/27). Only one sample (L/28, 22.97 ± 3.79) gave results significantly lower (p<0.001) than the control value (35.29 ± 4.67).

At the subsequent culture time of 96 h, a significant difference of the percentage of BrdU-positive cells in comparison to controls was observed in the cultures with the alloy L15/I (p < 0.05). In this case, the mean value was higher than that expressed by control cultures. In contrast, the other ones behaved in a way similar to controls, since no significant differences were found.

We have also evaluated the differences between the percentage of BrdU-positive cells in each culture with each dental alloy with that of the same alloy at the subsequent time. Interesting, a highly significant increase of the BrdU-positive cells was observed between the cultures in the presence of the alloy L15/I at 96 h as compared to the same alloys at 72 h. The percent values ranged from 26.48 ± 5.67 at 72 h to 42.13 ± 6.80 at 96 h (p < 0.01). The other fibroblast cultures behaved in a way very similar to controls, since no significant differences were found between the percentages of BrdU-positive cells at the two established culture times.

Fibronectin organization

Fibronectin showed three different organization levels, i.e. diffuse in the extracellular matrix, organized both in fibrils fibrillar and in focal adhesions. The results of the semiquantitative evaluation of fibronectin expression are summarized in Table 3, with ++++ indicating the maximum expression, + indicating a minimal expression and - represents no expression.

At 72 h, disperse fibronectin was observed in all examined samples, with more being expressed in cultures

<table>
<thead>
<tr>
<th>ALLOY</th>
<th>DISPERSIVE FIBRONECTIN</th>
<th>FIBRILLAR FIBRONECTIN</th>
<th>FOCAL ADHESIONS</th>
<th>CULTURE TIMES</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/15I</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96 h</td>
</tr>
<tr>
<td>L/27</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96 h</td>
</tr>
<tr>
<td>L/28</td>
<td>++++</td>
<td>-</td>
<td>+++</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96 h</td>
</tr>
<tr>
<td>γ</td>
<td>++++</td>
<td>+</td>
<td>++</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96 h</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96 h</td>
</tr>
</tbody>
</table>
together with the L/15I and L/28 alloys. The fibrillar organization appeared less evident than the previous organization level and was completely absent in cultures with the L/28 alloy. As far as focal adhesions were concerned, they were more present in the sample cultured with the alloy 7 as compared to the other alloys.

At 96 h, a scarce amount of diffuse fibronectin was observed in specimens grown with alloys L/15I (Fig. 1), L/27 (Fig. 2) and 7 (Fig. 4), as well as in the controls. On the contrary, fibronectin was more represented in the specimen grown with alloy L/28, in which a marked focal adhesion expression was also evident (Fig. 3). Fibroblast cultures with the alloys L/15I and L/27 expressed the most evident fibronectin fibrillar organization (Figs. 1 and 2), that was associated with pronounced focal adhesions. Cultures with the L/28 alloy expressed no fibrillar fibronectin also at 96 h, whereas well evident focal adhesions were present (Fig. 3).

**Discussion**

The evaluation of cell proliferation rates has been widely proposed as a useful tool for determining the biocompatibility in vitro of both dental implants (19, 20) and dental metal alloys (1,4,8-10,21). Several primary and transformed cell lines have been used to develop in vitro systems to study cell interactions with biomaterials. However, primary human cell culture systems, i.e. human gingival fibroblasts, do not always exhibit reproducible
results (14). Therefore, in the present study, we employed a stabilized cell line (Flow 2002 fibroblast), since previous reports on the biocompatibility of biomaterials had also indicated this experimental method in vitro (1,4,8,20). We performed the BrdU-immunocytochemical technique for detecting proliferating cells in the S-phase, since this method is easier to perform than the radiisotopic technique involving tritiated thymidine incorporation, which requires prolonged experimental times (12). Using the same immunocytochemical method, as was used in a previous investigation (4) we examined the biocompatibility of six single-phase dental casting alloys by determining cell proliferation rates correlated to fibronectin arrangement in human fibroblast cultures, at more prolonged culture times up to 168 h. We concluded that cells cultured in the presence of alloys containing the highest amount of Au (expressed in at%) behaved in a way similar to control cultures, above all at longer culture times (120 and 168 h), whereas the other five exhibited different behaviors.

In the present study, we limited the observation period by considering only two culture times, i.e. 72 and 96 h, in accord with other reports (2,8-10), since cell behaviors were already different in the presence of the different alloy at these two shorter culture times.

In 1990, Craig and Hanks (1) classified twenty-nine dental casting alloys into five biocompatibility grades, that depended upon their metal composition. Grade 1 (high Au and/or Pd content and absence of Cu) represented the most biocompatible alloys, whereas grade 5 represented the most cytotoxic ones. Grades 2, 3, 4 included alloys which had progressively lower biocompatibility.

As far as the dental metal alloys tested in this work were concerned, L15/I, L27 and γ may belong to grade 2, whereas L28 may belong to grade 3. Considering data related to BrdU incorporation in cultures with alloy L15/I we found the highest percentage of BrdU-positive cells after 96 h. This alloy contains the highest percentage of noble elements (84.09 at% Au+Pt+Pd) and does not contain any amount of Ag, therefore this cell behavior was expected and was in accord with the evidence of our previous investigation (4). Also cultures with alloy L27 exhibited a high percentage of BrdU-positive fibroblasts with no significant difference as compared to controls. L27 alloy also had a high content of noble elements (76.02 at% Au+Pt+Pd), but with a prevalence of Pd (74.50 at%) in comparison to Au and Pd. Although it also contained Ga, that could be certainly released into the culture medium (2), as well as Ag (1.84 at%), we did not observe any significant different behavior as compared to controls at 96 h.

The behavior of the fibroblasts grown with the L27 alloy was similar to that observed in presence of the alloy γ. When fibroblasts were grown in presence of the alloy L28, a highly significant decrease of BrdU-positive cells (p < 0.001) was observed as compared to controls. L28 contains the highest Ag percentage (32.60 at%) and also has the lowest amount of noble elements, that were represented by only Pd (58.70%). These findings are in accord with previous observations on fibroblast cultures in presence of dental casting alloys (4): in fact, dental casting alloys with higher Ag content and lower amounts of noble metals had lower proliferation activity. It was also in accord with Craig and Hanks (1), who found a high cytotoxic activity in cultures with alloys containing from 20 to 35 at% Ag. Moreover, Wataha et al. have found that Ag+ could be released into the culture medium from alloys containing Ag. Further, they stated that a correlation exists between the at% of Ag and Ag+ released in the media, where this amount is dependent on a second-order polynomial relation, if the at% of Ag is higher than 10% in single phase alloys (2).

We should also consider that three of the alloys presently tested contain Cu, i.e. L15/I, L27 and γ. In vitro studies on copper-based dental alloys, with a Cu weight percentage between 76% and 87% found, altered cell behavior in immunocompetent cells (22). However, Cu amounts in these three alloys are much lower than in a copper based one, therefore it is probable that these Cu percentages (15.00, 6.32 and 10.30 at%) could not influence cell proliferation.

In considering the influence of the presence of Au in the four tested dental alloys, we observed that fibroblasts cultured in the presence of the alloy L15/I, with a composition of grade 2 in accord to Craig and Hanks (1), the highest Au content among the tested alloys, a high content of noble elements (Au+Pd+Pt 93.60 wt%, 84.09 at%), 15 at% Cu, and no Ag, had the highest percentage of BrdU-positive cells at 96 h. Further, the only significant (p < 0.05) increase of this value was between 72 h and 96 h. This behavior was not so marked for the cells grown together with the alloys L27 and γ, perhaps since these also contain Ag in their elemental composition.

On these bases, we assume that Au is effective in maintaining cell viability, when present at over 71 at% (1, 23); however, we showed in this work that it could also be true when Au appears in a lower amount, i.e. in alloy γ (69.80 at%) and this evidence is also supported by Craig and Hanks who have previously reported that high percentages of noble elements (between 65 and 94 at%) in alloys with 25-27 at% of Ag and no Cu could enhance their biocompatibility (1).

The organization of the extracellular matrix protein...
fibronectin was also investigated by immunocytochemical techniques as a parameter for the evaluation of cell adhesion capability. Fibronectin could appear diffuse in the extracellular matrix, arranged in fibrils or organized in focal adhesions. Available evidence suggests that each fibronectin organization is strictly related to cell proliferation (13): in particular, fibronectin organized in focal adhesions enhances cell entry into the S-phase and therefore stimulates the growth cycle. On the other hand, we have previously shown that fibronectin arranged in focal adhesions had a better biocompatibility of a biomaterial, since fibronectin organization was correlated to cell proliferation rates (4). However, in the present investigation we found that the alloy in which there was a prevalent fibronectin arrangement in focal adhesions (L/28) at 96 h did not present the highest cell proliferation rate expressed as percentage of BrdU-positive cells at the same culture time. Evidence from this investigation suggests that it is necessary for not only the expression of focal adhesions, but also of fibronectin fibrils. The expression of both fibrils and focal adhesions was observed in 96-h cultures in the presence of the alloys L15/I, L/27 and y, whereas focal adhesions were associated to disperse fibronectin and the complete absence of fibrillar fibronectin both at 72 and at 96 h in the presence of the L/28 alloy. By relating these data to the percentages of BrdU-positive cells at 96 h in the presence of the L/28 alloy, the arrangement of fibronectin was related to a significantly lower value of fibroblast in the S-phase. On the contrary, the fibronectin arrangements in the presence of alloy L15/I were similar to the controls at the chosen culture times with a prevalence of focal adhesion associated to fibrils. On the basis of these findings, this alloy could be considered the most biocompatible of those examined.

The present investigation provides useful information on the relationship between cell proliferation rates and fibronectin organization. Fibronectin arranged in focal adhesions enhances cell proliferation, but it is not sufficient. It is also necessary the expression of fibronectin fibrils.

We conclude that alloys inducing a predominance of fibronectin organized in focal adhesions and in fibrils could be the most biocompatible. The observation of fibronectin organization could surely be a useful tool to determine the biocompatibility of dental metal alloys, as well as of other biomaterials.

**Acknowledgments**

The authors wish to thank Mrs. Giovanna Baldini for technical assistance.

This work was supported by grants from the Italian MURST (40% and 60%, University of Trieste, Italy).

**References**


