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Comparison of visually controlled and automatic histomorphometric evaluation of soft tissue

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For histomorphometric evaluation of human soft tissue a visually controlled method for determination and counting of Giemsa stained histological structures and an automatic image analysis after monoclonal staining were applied in order to compare the two evaluation systems. Human tissue adjacent to commercially pure titanium fracture fixation plates was obtained during routine removal of the plates after 1.5 years of implantation. Tissue samples of 20 patients were examined with both techniques. For monoclonal staining the following antibodies were used: HAM 56 (macrophages), propyl-4-hydroxylase (fibroblasts), CD 20 (B-lymphocytes), CD 2 (T-lymphocytes), CD 25 (activated T-lymphocytes), CD 4 (T-helper/inducer cells), CD 8 (T-suppressor/cytotoxic cells). For the visually controlled evaluation, we observed connective tissue cells (fibrocytes, fibroblasts) $709 \pm 19/mm^2$ (mean $\pm$ SEM per mm^2), macrophages $4.0 \pm 0.9/mm^2$ and lymphocytes $48.9 \pm 15/mm^2$. With image analysis we found fibroblasts $223 \pm 23/mm^2$, macrophages (HAM 56) $112 \pm 16/mm^2$, T-lymphocytes (CD2) $100 \pm 10/mm^2$, activated T-lymphocytes (CD25) $49 \pm 6/mm^2$. No B-lymphocytes were observed in the tissue samples. The results for macrophages ($p = 0.0001$) and lymphocytes ($p = 0.0001$) are statistically significantly higher using the image analysis. The antibody staining method in combination with an image analysis is to be recommended because of the time saved and the more precise identification of cells. Whenever no specific antibodies are available for the structure to be analysed, as in many animal studies, the visual method should be applied.

1. Introduction

The histomorphometric evaluation of the soft tissue adjacent to implants has been carried out for many years by visual control and counting of histological structures according to their morphology and their staining behaviour [1–5]. This method is time consuming and it is difficult to determine some cell types without special staining techniques (macrophages without phagocysed material, fibroblasts versus fibrocytes). Some cell types are not distinguishable without selective staining (T-lymphocytes versus B-lymphocytes, subgroups of T-lymphocytes). Since monoclonal antibodies are available for many specific cell types, especially in human cells, this technique can be used for selective cell staining [6]. Provided a selective staining of the cell types is used, an image analysis system can be applied for counting the histological structures [7].

The soft tissue surrounding commercially pure titanium (cp Ti) fracture fixation plates [8] was analysed in a prospective clinical study. The standard staining method using visually controlled determination and counting of the cell types, and the monoclonal staining method with automatic image analysis were applied to the same samples in order to compare the evaluation systems. We were interested in a comparison of the quantitative results and the time consumed using the two methods. Additionally, monoclonal staining should give information about the subgroups of some cell populations.

2. Experimental method

Human tissue in contact with a commercially pure titanium fracture fixation plate [8] was obtained during routine removal of the plates after 1.5 years of implantation. Tissue samples from 20 patients were examined. For monoclonal staining the tissue samples were removed, frozen, embedded in cryo-m-bed (Bright, UK), serial sectioned at 7 μm, and stained using the following anti human antibodies: HAM 56 (macrophages, some endothelial cells and monocytes), propyl-4-hydroxylase (fibroblasts), CD 20 (B-lymphocytes), CD 2 (T-lymphocytes), CD 25 (activated T-lymphocytes), CD 4 (T-helper/inducer cells), CD 8 (T-suppressor/cytotoxic cells). Each individual section

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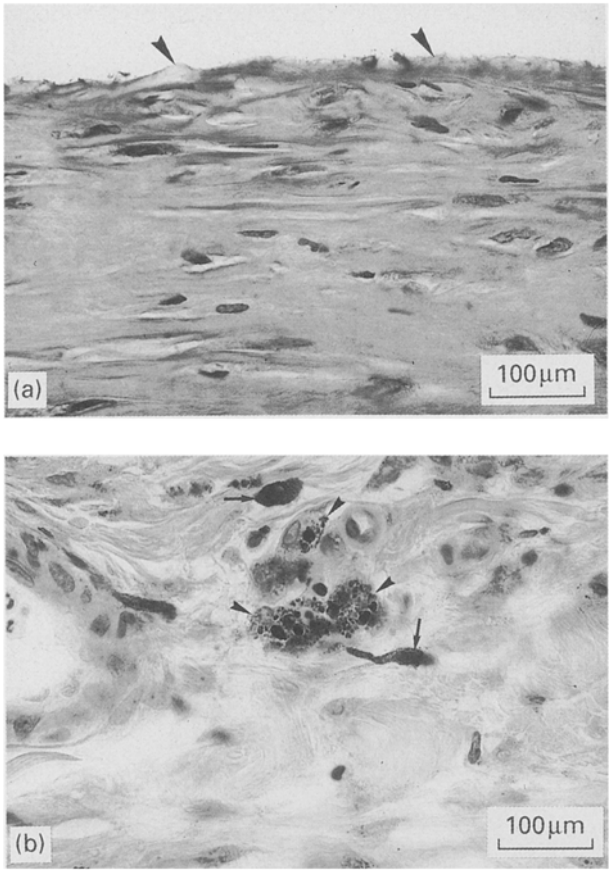

Figure 1 Light micrographs of Giemsa stained and visually evaluated tissue. (a) Connective tissue cells (fibroblasts, fibrocytes) present close to the implant tissue interface (arrow heads). (b) Macrophages with phagocysed material (arrow heads) and mast cells (arrows). Both cell types are frequently seen in the vicinity of blood vessels.

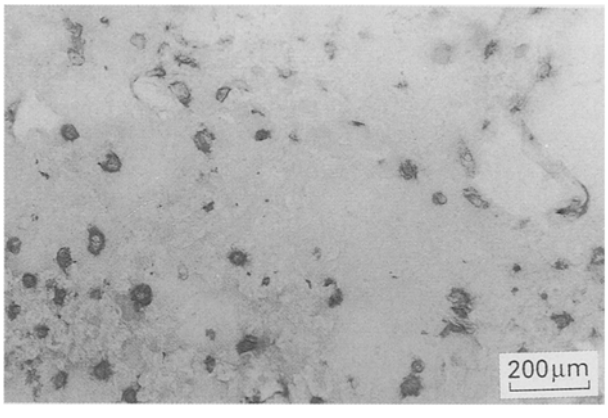

Figure 2 Light micrograph of monoclonal stained macrophages used for image analysis (HAM 56).

was then analysed using an automatic image analysis system, with routines written specifically to select cells stained with these antibodies at a magnification of $\times 200$. The tissue samples for the visually controlled evaluation were fixed in 4% buffered formol, dehydrated, embedded in methylmethacrylate, cut at 7 µm, Giemsa stained, mounted on glass slides, and evaluated in the light microscope at a magnification of $\times 400$. The cell types were differentiated according to their morphology and staining behaviour [10]: connective tissue cells (fibroblasts, fibrocytes), macrophages, round cells (lymphocytes, plasma cells). The number of microscopic fields which had to be evaluated in each sample was calculated by statistical methods [4, 9]. On average 20 to 40 microscopic fields were evaluated in each sample, depending on the heterogeneity of the histological structures. The two-sided standard t-test was used to calculate the significance of data differences ($p \leq 0.05$).

3. Results

For the Giemsa stained samples and visually controlled evaluation method, we observed connective tissue cells $709 \pm 19/mm^2$ (mean $\pm$ SEM per mm^2), macrophages $4.0 \pm 0.9/mm^2$, and round cells $48.9 \pm 15/mm^2$. With the antibody staining technique and automatic image analysis we found fibroblasts $223 \pm 23/mm^2$, macrophages (HAM 56) $112 \pm 16/mm^2$, T-lymphocytes (CD 2) $100 \pm 10/mm^2$, activated T-lymphocytes (CD 25) $49 \pm 6/mm^2$. T-helper cells (CD4) were found in all patients but one, with an average of $52 \pm 6/mm^2$; T-suppressor cells (CD8) were found in only nine patients, with an average of $32 \pm 11/mm^2$. No B-lymphocytes were observed in the tissue samples. The results for macrophages and lymphocytes are significantly higher using monoclonal staining and fully automatic image analysis (macrophages $p = 0.0001$, T-lymphocytes (CD2) $p = 0.0001$).

4. Discussion

The standard staining method with visually controlled determination and counting of cells is time consuming and has to be done by a histologically trained person. The evaluation of one sample took on average 3–4 h using the semiautomatic evaluation system [4]. Using the automatic image analysis system, the time taken to count the cells on a slide is dependent on the number of cells on the slide and the increasing complexity of the binary image with increasing cell number. This can result in counting times up to 3–4 h, however, no user supervision or input is required during this period and the system can be set up to run samples 24 h per day.

The results obtained by the parallel use of the two methods for the same samples allows some interesting observations. With the visually controlled method we counted fibroblasts and fibrocytes in one group, because they are difficult to differentiate. With selective staining of fibroblasts by immunological methods, the population can be counted separately. In our series the number of fibroblasts was found to be about one-third of the connective tissue cells (fibrocytes and fibroblasts). The proportion of fibroblasts in the population of CTCs may give information about dynamic cellular reactions in the connective tissue, since the fibre and extracellular matrix are mainly produced by fibroblasts [10].

Round cells are observed in the tissue adjacent to the implant. Using the visually controlled method, the number of round cells is half the result obtained by

antibody staining and automatic image analysis. The counting of round cells is relatively easy under visual control, so we suppose (under the precondition that no cross-staining occurred using the antibodies) a certain overcounting occurred with the automatic image analysis. With selective antibody staining the round cells could be identified as T-lymphocytes, and coupled with the presence of macrophages, the observed tissue reaction can be described as a chronic granulomatous inflammatory reaction. The fact that a large proportion of the lymphocytes are activated (CD25), indicates that there is some continued stimulation. From the presence of CD4 and CD8 positive cells a delayed hypersensitivity reaction cannot be excluded.

The results for the macrophages obtained by both methods are so different, that a comparison seems impossible. On the one hand, macrophages without phagocysed materials are difficult to determine without selective staining, therefore an undercount could be expected using Giemsa staining and the visually controlled method. On the other hand, the anti-human antibody HAM56 is not selective for macrophages, because endothelial cells of small blood vessels and monocytes are stained also, and an overcount may result. Both factors may explain the huge difference in the results.

5. Conclusions

- For biocompatibility testing in clinical studies the antibody staining method in combination with a fully automatic image analysis is to be recommended, because of the time saved and the more precise identification of cells.
- Whenever no specific antibodies are available for the structure to be analysed, as in many animal studies, the visual method should be applied.

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