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Quantification of the bone-related mRNAs at the bone/prosthetic interface

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Aseptic loosening is one of the major reasons for failure of joint prostheses. The periprosthetic tissue has previously been described microscopically; however, little work has been devoted towards quantitating genes expressed by cells at the materials/tissue interface. This study aims to characterize the phenotypic expression of osteoblasts and test the feasibility of quantifying the level of gene expression in periprosthetic tissue sections by combining *in situ* hybridization and image analysis techniques. There are many factors to consider when quantifying mRNA, in that comparing labeling between different cDNA probes, these should have comparable length and base comparison. The probes should be labeled with the same specific activity, that is the amount of probe to label added is the same, both between different probes and between batches of the same probe. Chromagen color reactions are variable in that the color development is not always linear and more likely follows a sigmoidal curve. Samples should only be compared when it is known that the reaction has been in the linear range. The image analysis of such staining introduces further factors which should be considered and controlled. Color analysis is a very complex problem with respect to reproducibly analyzing histological sections. The brightness component of the image should be independent of the colors within the image, in conventional RGB (red, green and blue) signalling mode this is not possible, while when using HSI (hue saturation and intensity) mode this becomes possible, and factors like staining intensity and brightness of the image become much more accountable and controllable. With these factors identified, we consider that the quantitative image analysis approach does allow comparison of patterns of bone-related mRNAs and demonstrates differences in expression in these osteogenic factors depending on distance from the prosthesis, tissue type, patient and device.

1. Introduction

Orthopaedic prosthetic joints are routinely accepted as a long-term treatment for chronic disabling forms of arthritis. However, a significant percentage of these devices will loosen, on average, after 10 y. The cellular response and the mediators stimulated to be produced and then released by cells, determines the fate of the implanted prosthesis. Periprosthetic tissue has previously been described histopathologically [1, 2], and also in terms of the cytokines involved [3], but there has been little work devoted towards quantitatively identifying cells expressing osteogenic parameters in this tissue, although some subjective evaluation has been performed [4]. The influence of the local environment on gene expression in cells at the prosthetic material/tissue interface has yet to be characterized in a repeatable and objective manner.

There are now many techniques available to probe in detail the osteogenic processes in a cellular response, immunohistochemistry, *in situ* hybridization and polymerase chain reaction (PCR) to name only three, the former two, in analysis, retaining a traditional histopathological presentation to identifying the mechanisms in that these can be performed on sections of tissue and examined using light microscopy. In probing the cellular mechanisms, it is paramount that techniques should focus on reproducibility and objectivity.

The aim of the present study was to utilize quantitative image analysis techniques to determine the expression of osteoblastic genes in the perioprosthetic

fibro-vascular tissue derived from aseptically loosened and failed prostheses. The following genes known to be part of the osteoid were studied, including alkaline phosphatase, thrombospondin, type I collagen, osteopontin, osteonectin, osteocalcin and bone sialoprotein. The work focuses on testing the feasibility of quantifying gene expression in tissue sections and determining if reproducible and meaningful data can be obtained by combining these two techniques.

2. Materials and methods

The cellular and tissue components of revised periprosthetic tissue were characterized using molecular biology and quantitative image analysis. Specimens of the bone–implant interface on the femoral, acetabular, or tibial side obtained during revision surgery were used in this study. Six patients who had osteolysis-associated loosening, were studied. The initial diagnosis that led to joint replacement was osteoarthritis in four patients, avascular necrosis of the stem in one patient, and slipped epiphysis in one patient. On revision there was a radiolucent line indicative of osteolysis around the implant. Five specimens came from joints with cemented prostheses, and one specimen was obtained from a joint where cement was not used. The duration of the implant ranged from 3–15 y. Polymethylmethacrylate bone cement was used with the hip arthroplasties; the knee arthroplasty was uncemented.

2.1. Tissue processing

Tissue was fixed chilled (4 °C) in freshly prepared ice-cooled 4% paraformaldehyde, decalcified and embedded in paraffin for *in situ* hybridization studies. Photobiotin-labelled cDNA probes were applied to the cells to probe for the following mRNA molecules; alkaline phosphatase (ALP), thrombospondin (Tsp) (a gift from Dr Phil Hogg, UNSW, Australia), procollagen osteonectin (ON); osteopontin (OP); and osteocalcin (OC); fibronectin (Fn). β-actin was used as a positive control and respective vectors were used as negative controls. All the cDNA probes used in this study (unless otherwise stated) were generously provided by Dr Walsh, Brown University, USA. All probes tested were of similar length.

Biotin was detected using multiple applications of streptavidin and biotinylated alkaline phosphatase and subsequently visualized by the *p*-nitrophenyl phosphate assay method [5].

2.2. Image analysis

After preparation, sections were visualized using a Carl Zeiss Jena Jenaval microscope (magnification × 320) with a JVC KY F30 3CCD color video camera attached. From each section 20 images were taken and analyzed using an Apple Macintosh IIfx running Colourvision; 32 bit colour image processing software (Improvision, Coventry, UK). This software gives the user access to image manipulation and analysis parameters. These options can be linked into automated routines, enabling measurement parameters to be fixed and therefore remain constant over all sections analyzed.

Positive staining was colored pink to red and the cell nuclei were counterstained blue using haematoxylin; subsequent to capturing images in RGB (red, green, blue) mode the signalling mode was changed to HSI (hue saturation and intensity). The signalling mode hue, saturation and intensity was utilized to reduce the effects of variable stain intensities and other brightness factors. In this mode, the hue component in combination with saturation was used to define positive staining, nuclear staining and unstained tissue,

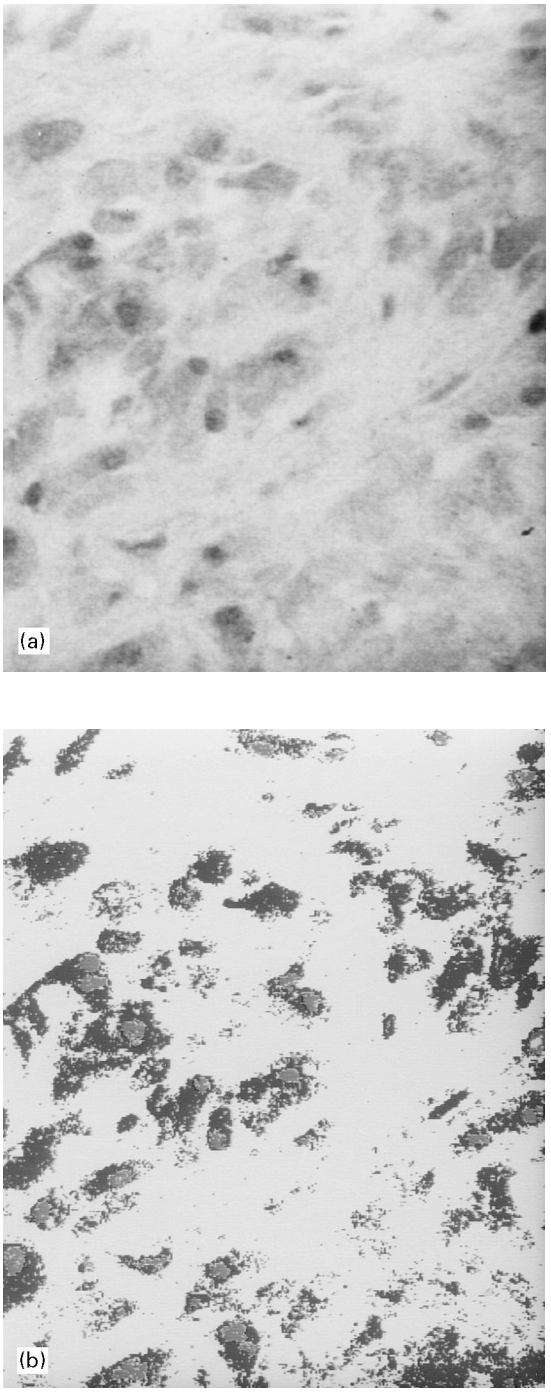

Figure 1 (a) Osteocalcin-stained section after capturing for image analysis, (× 25). (b) Binary image of image (a) after thresholding for positive stain and cell nuclei; the change in gray scales depict the two levels of thesholding, × 25.

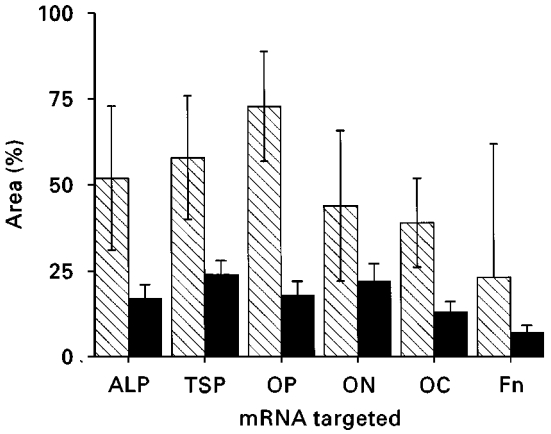

Figure 2 Quantitative image analysis of the bone-related mRNAs expressed by cells residing in the pseudosynovial membrane ▨ and in the bone of periprosthetic tissue ■. Alkaline phosphatase (ALP), thrombospondin (Tsp), osteonectin (ON), osteopontin (OP), osteocalcin (OC) and fibronectin (Fn).

reducing the significance of the brightness of the strain or the level of illumination, these factors being controlled in the intensity component of the signalling mode. The images were thresholded according to three fixed color ranges, each measured parameter being given a different color in the thresholded binary image. From the binary image the area of positive staining was measured; Fig. 1 demonstrates the selection of positive staining and nuclei (Fig. 1b) from the captured image (Fig. 1a).

Positive staining and cell nuclei were measured in global terms, the results being expressed as a percentage between cell nuclei and positive stain and as percentages of the total tissue area measured within each field.

3. Results

Quantitative image analysis of the tissue retrieved from an aseptic loosened prosthesis is presented in Fig. 2. Results obtained from the different zones of the periprosthetic tissue showed that there are differences in expression for the various mRNA molecules tested, and this was dependent on the type of tissue, implant location and patient. Cells residing in the pseudosynovial membrane displayed strong expression of mRNAs, specifically for alkaline phosphatase, thrombospondin, osteopontin, osteonectin, osteocalcin and fibronectin. In contrast, the expression of these mRNAs was lower in bone, the expression in bone being measured from isolated nodules of bone embedded in the fibrous tissue zone. These differences between the two zones were very obvious, with the difference in osteopontin expression being the clearest.

4. Discussion

Analyzing a panel of *in situ* hybridization probes is questionable and problematical, being hindered by a number of technical considerations. The key factors to consider are given below.

1. To compare labeling between different probes, there is a need to ensure that they have a comparable length and base composition and as much as possible that they will anneal or stick to the target DNA with comparable kinetics and at the same temperature. If they have similar length and base composition, this should be acceptable.

2. The probes should be labeled with the same specific activity, that is the amount of probe to label added is the same, both between different probes and between batches of the same probe. Because labeling of probes may not be to the same specific activity, the use of a standard curve is required to enable the quantitative comparison between different probes.

3. Chromagen color reactions are variable, in that the color development is not always linear and more likely follows a sigmoidal curve. Samples should only be compared when it is known that the reaction has been in the linear range. This is very difficult to ascertain and does not take into account all the variations in day-to-day staining intensity observed with this type of procedure.

Color analysis is technically very complex with respect to reproducibly analyzing histological sections with counterstain. Conventionally, images are visualized in the RGB (red, green and blue) signalling mode in which each color component is fundamentally linked to the intensity or brightness of the image. By using an alternative signalling mode, HSV (hue, saturation and volume) more conventionally referred to as HSI, hue saturation and intensity the brightness of the image can be separated from the color. The true color component comprising of hue and saturation and the brightness or illumination being controlled in the intensity component, this enables the system to reproducibly detect colors that have been defined as the colors specific to the stains for analysis.

In isolation this does not totally remove the constraints of illumination and variation in intensity due to the staining procedure; however, these systems are sensitive analytical tools and, with the inclusion of staining controls to clearly determine the standard of the result from the staining procedure, the variables can be controlled and calibrated, clearly confining the protocol within strict analysis constraints.

The results for expression of mRNA do not demonstrate any consistency at this stage, as the sample number is still very low for any prevalent characteristics to emerge and be proven statistically. There are clearly large differences in expression in changing the type of tissue analyzed and in changing the patient and the implant site but how significant these changes are in determining specific mechanisms is still unresolved, but may be only through a lack of data. It is clear that differences exist which, with additional samples, may prove to determine mechanisms in a statistically significant way.

5. Conclusion

This work has shown that active osteogenesis is taking place and that the expression of mRNA changes with tissue analyzed, device, implant site. Although tempting to speculate that these changes are related to bone formation and therefore may be an important

component of aseptic loosening and as such should be explored, because the study is at the preliminary feasibility stage, the number of samples is too small and too varied to confirm statistically, and therefore attribute real significance.

Despite these identified short-comings, we consider that the quantitative image analysis approach does allow, within microscopic sections, comparison of patterns of bone-related mRNA expression, as long as strict constraints on probe synthesis and application are applied as well as application of controls at the analysis stage. The application of a reproducible technique enables a long-term analysis of tissue as it becomes available to add to data and statistics.

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