The use of molecular biology in evaluating human wound healing

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Abstract

Proteins fulfil the majority of structural and functional requirements in living cells – medicinal drugs act directly or indirectly through proteins in the cell membrane or proteins located within the cell. The unique properties of a protein are determined by the sequence of amino acids within this macromolecule. The unit of deoxyribonucleic acid (DNA) that codes for a protein is known as a gene and is copied or transcribed to generate a gene transcript which specifies the sequence of amino acids in the protein.

The study of genomic DNA, gene transcripts and proteins at the molecular level is encompassed by the term molecular biology. This article will address the application of a recent advance in molecular biology, namely DNA microarray technology, to the evaluation of wound healing.

Introduction

In the last 3 decades there has been an enormous surge in basic scientific understanding of how biological systems work following key technical advances. For example, although the basis of the genetic code in deoxyribonucleic acid (DNA) was described by Watson & Crick in 1953, it took 2 more decades before scientists could begin to make sense of the long stretches of DNA which make up the human genome.

The discovery of two types of enzyme was crucial in this next advance. Restriction enzymes were able to catalyse the cutting of DNA at specific sites into more workable lengths and ligases were able to join bits of DNA together again. This enabled the insertion of DNA restriction fragments into naturally replicating DNA vectors called plasmids and gave scientists the capacity to amplify the DNA fragments to amounts that could be used for further analyses. These analyses were possible because procedures were also developed at that time that could elucidate the sequence of nucleic acids in DNA fragments up to 500 nucleotides in length.

Ultimately, these strategies, together with another technical advance in the form of the polymerase chain reaction (PCR), have led to the sequencing of the entire human genome, a scientific feat which compares with any made in the last century.

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DNA microarray technology

With the sequencing of the human genome and the development of another break through technology, namely DNA microarrays, biological scientists will have the capacity to look at global gene expression for the first time. In other words, they will have a measure of the expression of every single gene within a cell type at any particular time. Until commercial DNA microarrays became available, scientists could only examine a small number of genes at any one time. This set severe restrictions in terms of interpretation of results. An analogy would be that of an artist painting a nude when all he could see was the model's little toe. He may have painted the toe accurately but the overall figure represented

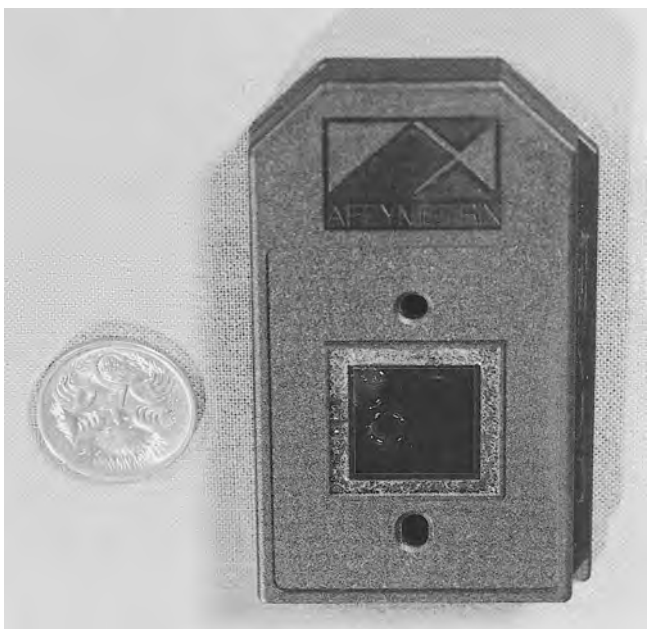
his imaginary vision and was therefore scientifically highly inaccurate (although possibly quite beautiful).

DNA microarray technology is potentially the most powerful tool available in the field of molecular biology over the next few years. Microarrays work on the exquisite specificity of complementary base pairing so that genes or parts of genes called oligonucleotides, when immobilised on the microarrays, will bind to the same sequence in gene transcripts from cell extracts.

There are several different types of DNA microarrays currently available. One approach has printed DNA sequences on glass microscope slides using a robotic arrayer. Another has immobilised DNA fragments on a nylon membrane and a third has synthesised oligonucleotides on a silicon chip using photochemical techniques. The latter product has been developed by Affymetrix and can analyse the expressions of 12,000 genes using a chip the size of a 5 cent coin (Figure 1). The nylon microarray contains 4,400 DNA fragments and is the size of a credit card.

All microarrays are subjected to hybridisation reactions where the DNA spots can be exposed to fluorescence labelled or radioactivity labelled gene transcript products under conditions where complementary base pairing can occur. After hybridisation, measurement of fluorescence or radioactivity at each DNA spot provides an estimate of the amount of that particular transcript.

Figure 1. Front view of an Affymetrix chip that can detect expression of 12,000 individual human gene sequences. The rectangular chamber contains hybridisation solution and several air bubbles are visible (broken white outlines).



The technology requires high quality gene transcripts (ribonucleic acid, RNA) as starting material. The only source of RNA is living cells (cells from whole blood or cultured cells) or a fresh tissue biopsy. Depending on the type of DNA microarray technology chosen, the amount of RNA needed for a hybridisation run can be up to 10µg of total RNA which corresponds to 10mg wet weight of tissue (a 6mm punch biopsy generates 80-100mg wet weight of tissue).

Defective cutaneous wound healing in venous leg ulcers

Current understanding of wound healing in tissues, including skin, has identified three physiological phases – inflammation, tissue formation and tissue remodelling [1]. The phases of wound healing described so far are not achieved in chronic wounds.

One chronic wound that represents an enormous economic burden to the health system is the venous leg ulcer. Treatment of venous leg ulcers accounts for 1-2 per cent of the health care budgets of many western countries [2,3]. Although a condition associated with old age, a significant percentage of patients have their first ulcer during their working lives. The duration of individual leg ulcers is very long (>6 months in 46 per cent of patients) and a high rate of recurrence is a feature of chronic venous leg ulcers [3]. For many patients (47 per cent), ulceration has been occurring for over 5 years [4] and has a profound effect on quality of life.

It is generally agreed that the underlying problem in venous leg ulceration is a diseased venous system in the affected leg [3,5,6]. As a result, venous pressure in the deep veins within the leg does not fall much during ambulation, whereas normally there is a transient fall in venous pressure following emptying. The sustained ambulatory pressure in the diseased system is called venous hypertension and the mechanisms by which this leads to venous leg ulceration are a point of contention. A number of hypotheses have been put forward in an effort to understand the causes of venous ulcers but generally these have difficulty in explaining the sustained inflammatory state that is a characteristic feature of venous leg ulcers [7].

General considerations in application of DNA microarrays

It is anticipated that the utilisation of DNA microarray technology will generate exciting new findings in human

venous disease and clear up some of the controversy regarding the mechanisms underlying venous leg ulceration.

Dr Richard Young has recently reviewed application of DNA microarrays to other biomedical areas and has observed that expression profiles provide a molecular phenotype or a signature for cells within a particular perturbed environment [8]. For example, Young cited work which presented two dimensional hierarchical clustering of gene expression profiles, reflecting a "cholesterol-deficient phenotype". The expression profile signature for cells with diminished ability to synthesise cholesterol was observed, whether due to treatment with the drug lovastatin or due to genetically reduced levels of HMG-CoA reductase [9].

More evidence of gene clustering has been provided by a number of investigations of co-regulated genes involved in cell cycle progression in aged fibroblasts [10, 11]. These studies point to an experimental approach for determining the mechanisms affected by uncharacterised pathologies such as defective wound healing, namely the matching of the pathological profile with profiles resulting from known disturbances of cellular pathways [8, 9]. It should be possible to obtain signatures – for example for an 'oxidative stress phenotype' or a particular 'activated leucocyte phenotype' – and apply this to understanding of the formation of venous leg ulcers.

Successful application of DNA microarrays to classification of cancers

Thus far, the most successful application of DNA microarray technology has been to enable classification of leukaemias [12] and diffuse large B-cell lymphoma [13]. In the past, acute leukaemias were classified into those arising from lymphoid precursors (ALL) or from myeloid precursors (AML) by interpretation of a range of diagnostic tests. Unfortunately, classification was imprecise, and errors had serious implications in terms of the cure rate and in prevention of unwarranted toxicities due to inappropriate drug therapies.

Application of DNA microarrays to a panel of known acute leukaemia samples enabled Golub *et al.* [12] to identify a set of genes most closely correlated to AML-ALL discrimination. An assessment using 50 genes from this set was applied to an independent panel of leukaemia samples and resulted in distinction of AML-ALL with a 100 per cent accuracy.

Diffuse large B-cell lymphomas are also clinically heterogeneous, with 40 per cent of patients responding well to current therapy and having a good survival, the others succumbing to the disease [13]. Alizadeth *et al.* [13] were able to show that gene expression profiles or signatures reflecting a "germinal centre B-like phenotype" were associated with a significantly better overall patient survival.

It remains to be seen whether taxonomy of more common cancers such as breast and colon cancers will be possible from gene expression profile analyses of tumour biopsies.

Application of DNA microarrays to cutaneous wound repair

In vitro studies

Kessler *et al.* [14] have used microchip microarray technology to identify mechano-responsive genes in cultured skin fibroblasts subjected to radial and circumferential mechanical strain. Their model replicated the tensile forces that fibroblasts develop against granulation tissue matrix in wounds in order to initiate wound closure.

These authors identified 57 genes of known function (out of 7,100 on the microchip) that were induced at least 2 fold in mechanically stressed fibroblasts. Up regulated genes were placed in groups including those coding for proteins involved in proliferation and genes coding for extracellular matrix proteins, growth factors, protease inhibitors and components of the cytoskeleton [14]. However, Kessler *et al.* found that the tension induced 'matrix-synthesising' fibroblast had lower levels of expressions for genes related to inflammation such as Il-6. It would seem unlikely, therefore, that this particular phenotype of activated fibroblast contributes greatly to venous leg ulceration, the pathology of which is characterised by high levels of inflammatory cytokines in the wound environment [7].

Of greater relevance to venous ulcers is the study of Shelton *et al.* [15] who used microarray analysis to investigate senescence in skin fibroblasts. Senescent cells have shortened telomeres and are no longer able to replicate while exhibiting characteristic changes in morphology, including an enlarged cell size. Shelton and colleagues were able to demonstrate that increased expression of matrix degrading proteases (such as collagenase) and inflammatory chemokines and cytokines (such as MCP-1 and interleukin 1ß respectively) was a consistent trend at senescence [15]. Decreased expression of elastin, collagens and keratins (the latter two at higher serum concentrations) would also be likely to have deleterious effects on the extracellular matrix surrounding senescent cells [15].

Many of these biochemical changes seen by Shelton *et al. in vitro* are also observed in the wound environment of venous leg ulcers. For example, collagenase is consistently elevated (as are other matrix degrading proteases), inflammatory cytokines are increased [7] and there is abundant evidence of enzymes released by neutrophils (cells attracted into wound by chemokines). It is therefore of significant interest that the senescent phenotype can be induced by a metabolic insult that is a feature of venous ulcers, namely that of oxidative stress.

In vivo studies

Tsou *et al.* have used a commercial nylon microarray containing 4,400 DNA fragments in a study of gene expression in normal and hypertrophic scar biopsies compared to normal skin [16]. These researchers found that expression of a number of key collagens were significantly elevated in hypertrophic scars (IIIα1, Iα2 and IVα3). The only growth factor expression to be significantly increased in hypertrophic scars was IGF-2, a surprising finding bearing in mind the abundant literature implicating TGF-ß in the pathology of these lesions.

However, one must bear in mind that it is the bioavailability of TGF-ß *in situ* which determines its action and other control mechanisms may be in place. Expression of several keratins was diminished in hypertrophic scars (though not significantly), as was expression the tissue inhibitor of metalloproteinases, type I [1]. The latter finding was interpreted as supportive of a hypothesis of impaired matrix remodelling in hypertrophic scars [16].

Local experience with DNA microarrays

The Venous Leg Ulcer Research Laboratory at Fremantle Hospital has been involved in application of DNA microarray technology to biopsies collected from healing and non-healing venous leg ulcers. Initially we were concerned that RNA of sufficient quality could not be extracted from ulcer tissue. To date, however, we have extracted over 40 biopsies and have successfully utilised a number of RNA samples in runs with nylon membrane DNA microarrays. An example of data obtained in our laboratory is shown in Figure 2.

In broad terms, some of the observations made by us are similar to those made by Tsou *et al.* [16] in their publication. Although quite a few genes are up regulated for any particular patient, when one applies a cut-off of 2.0 and carries out statistical analysis for a series of patients, surprisingly few genes are identified, and these are mostly structural genes such as collagens and keratins. A cut-off of 2.0 may be too high for increased expression of a pivotal growth factor, receptor or transcriptional factor.

It is clear, however, that we are able to obtain expression profiles for venous leg ulcers and that there are few problems with application of nylon membrane DNA microarrays to this wound healing scenario. What we will be doing in the future is using the same RNA samples with more sophisticated DNA microarray chips (Affymetrix) in an ongoing collaboration with Pfizer UK. It is hoped that, by applying bioinformatics to the data generated by this study, our current state of knowledge of mechanisms underlying venous leg ulceration will expand substantially.

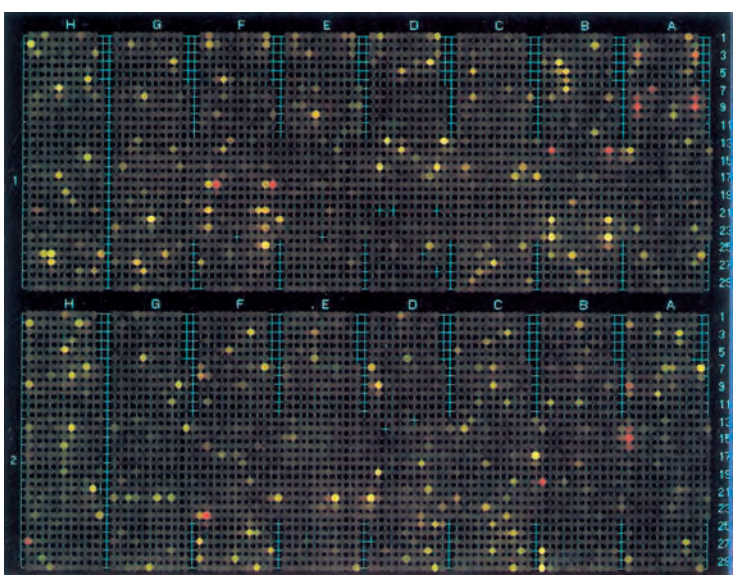


Figure 2. Comparison of gene expression profiles in pooled venous leg ulcer biopsies using nylon DNA microarrays. Biopsies from the non-healing base of the ulcer (92b) were compared to biopsies collected from a similar location after 2 weeks of bed rest when the base of the ulcer was now healing (85b). Genes which were elevated in non-healing ulcer base were bright green, genes which were highly expressed in healing ulcer base were bright red. Yellow and black spots reflected genes that were more evenly expressed in 85b/92b. The locations of spots on the grid provided identities of 4,400 individual genes.

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