RESEARCH COMMUNICATION

NADPH oxidase of chondrocytes contains an isoform of the gp91^{phox} subunit

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Previously it has been reported that chondrocytic cells produce oxygen free radicals and express the cytosolic components of NADPH oxidase. Here we report the expression of large subunit of the flavocytochrome of NADPH oxidase in chondrocytes and, further, show that the cDNA sequence contains three single base

pair differences compared with the phagocyte gp91^{phox} gene sequence. These base-pair differences may account for the different activity profiles reported between phagocytic and non-phagocytic cells.

INTRODUCTION

It has become clear in recent years that many cell types produce oxygen free radicals, and that the NADPH oxidase which was originally thought to be unique to phagocytic cells is expressed far more widely. Reports have shown the expression of various NADPH oxidase components in, for example, fibroblasts [1], human glomerular mesangial cells [2,3], chondrocytes [4,5] and carotid body cells [6].

NADPH oxidase consists of essentially five unique peptides (reviewed in [7]). A flavocytochrome consisting of two membrane-bound peptides of 22 kDa and 91 kDa (p22^{phox} and gp91^{phox} respectively) make up the redox pathway, containing binding sites for NADPH, flavin and haem. Further, activation of the oxidase requires the translocation to the membrane of three cytosolic components of 40 kDa, 47 kDa and 67 kDa (p40^{phox}, p47^{phox} and p67^{phox} respectively). Defects within the genes encoding the NADPH oxidase will manifest themselves as chronic granulomatous disease, where the host defence mounted by phagocytic cells is greatly impaired [8].

However, in 1993 Meier et al. [9] investigated the free radical release from both phagocytic and non-phagocytic cells in patients suffering from chronic granulomatous disease and concluded that NADPH oxidase must exist in isoenzyme forms, and, using immunological methods, suggested that the gp91^{phox} component was genetically and functionally distinct in the two cell types. Furthermore, Jones et al. [3] failed to detect the presence of gp91^{phox} in human mesangial cells, suggesting that, here too, subtle differences may exist within this gp91^{phox} subunit [10].

Recently, we have reported that articular chondrocytes and an immortalized chondrocytic cell line, C-20/A4, have the capacity to produce superoxide ions, and that they express the genes encoding the small subunit of the flavocytochrome along with the cytosolic components [4,5]. Here we report the expression of the large flavoprotein component gp91^{phox} by the immortalized cell line C-20/A4, as well as in chondrocytes derived from a patient undergoing joint-replacement therapy. Furthermore, on analysis of the cDNA sequence, this gp91^{phox} appears to be an isoform of the one reported previously [11].

EXPERIMENTAL

Culture conditions

The C-20/A4 human chondrocyte cell line was derived from juvenile costal chondrocytes from a specimen of rib cartilage which were immortalized using the origin-defective simian virus 40-containing large T antigen (SV40Tag) [12,13]. These cells were cultured as described previously [4]. Primary human chondrocytes were kindly supplied by Dr. B. Ashton (Leopold Muller Arthritis Research Centre, Robert Jones and Agnes Hunt National Health Service Trust, Oswestry, Shropshire, U.K.). These cells were obtained from a patient undergoing joint-replacement therapy and were cultured to confluency in a monolayer. The cells were harvested as described previously [4].

Preparation of total RNA, reverse transcriptase (RT)-PCR and sequencing

RNA preparation, cDNA production and PCR were done as described previously [4]. Sense and anti-sense primers were designed against the published mRNA sequence of the gp91^{phox} component from human neutrophils. The PCR products, generated using complementary primer pairs, were cloned and sequenced. The sequences (5'–3') of the PCR primers which were used to amplify the regions of chondrocyte cDNA, where sequence differences were observed are as follows. Primer set 1: sense, CCA ACT GGG ATA ATG AAT TC; antisense, TCC ACT GAC ATT ACT GAG AGG. Primer set 2: sense, CTA AGA TAG CGG TTG ATG GG; antisense, GAA TTC ATT ATC CCA GTT GG. Primer set 3: sense, GAA ACC CTC CTA TGA CTT GG; antisense, CCC ATC AAC CGC TAT CTT AG. PCR products were cloned using a TA cloning kit (Invitrogen). For each PCR product generated from the human chondrocyte line, two of the resulting clones were selected for sequencing. This procedure was then repeated on cDNA generated from freshly isolated RNA from the cell line to confirm any sequence differences between the published data for human neutrophil gp91^{phox} mRNA and the sequence data generated from the

Abbreviation used: RT, reverse transcriptase.

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C-20/A4 cell-derived cDNA. Clones generated from the chondrocyte cell line were sequenced using the Amplicycle Sequencing Kit (Perkin Elmer) with the universal primers provided, at least once in each direction, following the manufacturer's instructions.

To confirm that the sequence differences between neutrophil and chondrocyte were not just a feature of the cell line used, primary human chondrocytes were obtained from a patient undergoing joint-replacement therapy. The three PCR primer sets that amplified regions where sequence differences had been identified between the neutrophil and chondrocyte line sequence were used for PCR using cDNA derived from these cells. Clones of the PCR products of the three target regions were generated as described above, except that, in this case, the clones were sequenced commercially (MGW Biotech).

RESULTS AND DISCUSSION

Expression of gp91^{phox} in chondrocytic cells

Immortalized chondrocyte cells, C-20/A4, were cultured, harvested and total RNA prepared as described in the Experimental section. Using primers designed against the known phagocytic sequence of gp91^{phox}, RT-PCR was performed. A product of the predicted size (417 bp) using primer set 1 was obtained as shown in Figure 1(A). To investigate whether isolated chondrocytes from articular tissue also expressed gp91^{phox}, the procedure was repeated using cells from a patient undergoing joint-replacement therapy. Again, the PCR product of the predicted size (417 bp) was obtained using primer set 1 (Figure 1B), suggesting the expression of this component in these cells. This supports the previous evidence that NADPH oxidase subunits are expressed by chondrocytes [4,5], and that NADPH oxidase activity could be responsible for the oxygen free radical release by these cells.

Figure 1 RT-PCR amplification of chondrocyte-derived cDNA using primers against the known gp91^{phox} sequence

RNA was extracted from C-20/A4 cells and isolated chondrocytes and was used for the synthesis of cDNA as described in the Experimental section. PCR primer set 1 was used to probe for expression of gp91^{phox}. (A) C-20/A4 cells: lane 1, 100 bp ladder for markers; lane 2, RT-PCR product; lane 3, negative RT control (identical procedure but without RT). (B) Isolated primary chondrocytes: lane 1, 100 bp marker ladder; lane 2, RT-PCR product; lane 3, negative RT control (no RT).

Scheme 1 Amino acid changes seen in the chondrocytic gp91^{phox} cDNA sequences compared with published phagocyte sequences

RNA was extracted from C-20/A4 cells and isolated chondrocytes, cDNA was synthesized, PCR performed and the products were sequenced as described in the Experimental section. Sequences were aligned using the Genetics Computer Group software (Daresbury, Cheshire, U.K.). For each of the three regions, the top sequence is the published phagocyte sequence and the chondrocytic sequence is shown below. The arrows indicate the amino acid changes. Sequences of interest are shown in boxes and include, within Region 2, part of the NADPH-binding region [15] and, within Region 3, the p47^{phox}-binding region [17] and proposed flavin shield region [16].

Chondrocyte gp91^{phox} sequence analysis

To confirm that the PCR products obtained were indeed derived from the expression of a gp91^{phox} gene, the products were subsequently sequenced. However, previous reports [9,10] suggested that the gp91^{phox} gene product might be different in non-phagocytic cells when compared with the products obtained from phagocytic sources. To investigate this further, new PCR primers were designed so that the sequences obtained from the RT-PCR products first overlapped and secondly spanned the whole of the open reading frame proposed from the gp91^{phox} gene. Sequences were obtained from four clones from two different RNA isolations and the resulting sequence was aligned to the published sequence [11]. The sequence obtained from cDNA derived from the chondrocytic C-20/A4 cells was identical with that published previously except for, interestingly, three point differences: at position 884, T to C change; at position 1204, G to A change and at position 1690, a change of T to C.

These results confirm that a gp91^{phox}-like sequence is expressed by these cells and it is possible that the changes seen in the sequence were due to a polymorphism within the gene from the original donor from which the immortalized cells were derived. Although rare, such a polymorphism within this gene, at position 1102, has been described by others [14]. Therefore primary chondrocytes were obtained from a patient undergoing joint-replacement therapy and primers were designed which spanned the regions of interest containing the three proposed changes. RT-PCR was performed using cDNA derived from RNA isolated from these cells and the PCR products obtained were sequenced commercially. The sequences obtained were identical with the published phagocyte sequences, except for the same three changes seen at positions 884, 1204 and 1690.

The sequence data obtained from two independent chondrocytic sources, along with the fact that polymorphisms within this gene appear to be very rare, only one having been reported [14], suggest that chondrocytes express an isoform of the gp91^{phox} which has three base changes compared with the phagocytic sequence. The change of T to C at position 884 would mean the substitution of a valine with an alanine at amino-acid position 295 (Scheme 1). Analysis of the likely structural changes such a substitution would incur, suggest that, in fact, there would be little alteration. Furthermore, no functional activity has been assigned to this region of the polypeptide. However, the change of G to A at base position 1204 would mean the substitution of methionine for valine at amino-acid position 402 (Scheme 1). This is at the start of the proposed NADPH-binding site [15], where ⁴⁰²VVMLVGA... is predicted to be a conserved area within the gp91^{phox} sequence. Here, the first conserved valine would be methionine and, therefore, such a change could produce subtle but important changes in the functioning of the NADPH oxidase complex.

The third change seen was T instead of C at position 1690, which would result in the substitution of leucine for phenylalanine at amino-acid position 564 (Scheme 1). First, this is close to an area proposed to act as a flavin shield (Lys⁵⁶⁶ to Phe⁵⁶⁹) [16] but, secondly, this change lies within the site which is proposed to be involved in the interaction with the subunit p47^{phox} (Gly⁵⁵⁹ to Asn⁵⁶⁵) [17] and, therefore, will be involved in the activation of the enzyme complex. Here again, this transformation may cause subtle changes within the mechanism of the NADPH oxidase complex in these cells.

Several reports have presented data in which the activation and the activity of NADPH oxidase in non-phagocytic cells is surprisingly different to that reported for phagocytes. For example, Jones et al. [3] reported that the rate of superoxide release in stimulated mesangial cells was only 1–2% of that of monocytes, but that the release of superoxide continued over many hours. Fibroblasts also had a time course of release of oxygen free radicals over hours, with low maximal rates [18]. In porcine chondrocytes, no activation was seen on the addition of the phorbol ester PMA [5]. Here, for the first time, we present data which suggest that NADPH oxidase from phagocytic and non-phagocytic sources are different, and which could account for the activity differences reported by others. Clearly, the NADPH oxidase complex in difference cell types must have different functions and, therefore, the presence of isoforms of

NADPH-oxidase components could be exploited as targets for therapeutic agents. New inhibitors of free radical production could be targetted to specific isoforms where, for example, inflammatory activity of phagocytes needs to be suppressed without affecting the activity of NADPH oxidase in non-phagocytic cells.

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REFERENCES

- Meier, B., Cross, A. R., Hancock, J. T., Kaup, F. J. and Jones, O. T. G. (1991) *Biochem. J.* **275**, 241–245
- Radeke, H. H., Cross, A. R., Hancock, J. T., Jones, O. T. G., Nakamura, M. and Resch, K. (1991) *J. Biol. Chem.* **266**, 21025–21029
- Jones, S. A., Hancock, J. T., Jones, O. T. G., Neubauer, A. and Topley, N. (1995) *J. Am. Soc. Nephrol.* **5**, 1–9
- Moulton, P. J., Hiran, T. S., Goldring, M. B. and Hancock, J. T. (1997) *Br. J. Rheumatol.* **36**, 522–529
- Hiran, T. S., Moulton, P. J. and Hancock, J. T. (1997) *Free Radicals Biol. Med.* **23**, 736–743
- Kummer, W. and Acker, H. (1995) *J. Appl. Physiol.* **78**, 1904–1909
- Henderson, L. M. and Chappell, J. B. (1996) *Biochim. Biophys. Acta* **1273**, 87–107
- Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1995) *Biochim. Biophys. Acta* **1227**, 1–24
- Meier, B., Jesaitis, A. J., Emmendorfer, A., Roesler, J. and Quinn, M. T. (1993) *Biochem. J.* **289**, 481–486
- Jones, O. T. G., Jones, S. A. and Wood, J. D. (1995) *Protoplasma* **184**, 79–85
- Royer-Pokora, B., Kunkel, L. M., Monaco, A. P., Goff, S. C., Newburger, P. E., Baehner, R. L., Cole, F. S., Curnutte, J. T. and Orkin, S. H. (1986) *Nature (London)* **322**, 32–38
- Goldring, M. B., Birkhead, J. R., Suen, L. F. and Yamin, R. (1993) *Arthritis Rheum.* **36**, S49
- Goldring, M. B., Birkhead, J. R., Suen, L. F., Yamin, R. and Mizuno, S. (1994) *J. Clin. Invest.* **94**, 2307–2316
- Kuribayashi, F., de Boer, M., Leusen, J. H. W., Verhoven, A. J. and Roos, D. (1996) *Hum. Genet.* **97**, 611–613
- Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H. and Scrase, G. (1992) *Biochem. J.* **284**, 781–788
- Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L. and Kwong, C. H. (1992) *Science* **256**, 1459–1462
- De Leo, F. R., Ulman, K. V., Davis, A. R., Jutila, K. L. and Quinn, M. T. (1996) *J. Biol. Chem.* **271**, 17013–17020
- Meier, B., Radeke, H. H., Selle, S., Sies, H., Resch, K. and Habermehl, G. G. (1989) *Biochem. J.* **263**, 539–545