MURINE RETROVIRUS INFECTION AND THE EFFECT OF CHRONIC ALCOHOL CONSUMPTION: PROTEOMIC ANALYSIS OF CARDIAC PROTEIN EXPRESSION

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(Received 8 July 2002; first review notified 9 August 2002; in revised form 11 September 2002; accepted 28 September 2002)

Abstract — Aims: The cardiovascular complications of acquired immunodeficiency syndrome (AIDS) are serious, including the occurrence of pathological heart conditions such as cardiomyopathy. Chronic alcohol consumption accentuates the severity of AIDS and may contribute to the development of cardiomyopathy. The aim of this work was to use a proteomics approach to investigate global alterations in protein expression in a mouse model of AIDS in the presence or absence of chronic alcohol consumption. Methods: Cardiac proteins were separated by two-dimensional polyacrylamide gel electrophoresis and quantitative computer analysis was used to evaluate the resulting two-dimensional protein profiles. Proteins that were differentially expressed in the hearts of mice from the different experimental groups were identified by peptide mass fingerprinting by matrix-assisted laser desorption/ionization mass spectrometry. Results: A number of specific proteins were observed to be differentially expressed in the mouse heart due to the effect of ethanol feeding alone. Differentially expressed proteins were also observed that were due to viral infection alone. Ethanol feeding and viral infection appeared to have similar effects on the expression of a number of proteins. A total of 24 proteins were altered by infection alone. Of these 24 proteins, eight were affected by alcohol, with six alterations being ameliorated and two being exacerbated by alcohol. Two of these proteins have been identified as the 27 kDa heat-shock protein and mitochondrial long-chain acyl-CoA thioesterase 1. Conclusions: These results suggest that chronic alcohol consumption may exacerbate the effects of viral infection on the heart by lowering the stress response leading to de-protection and further cytotoxic effects.

INTRODUCTION

In this study, we used a proteomic approach to investigate a murine model of acquired immunodeficiency syndrome (AIDS) induced by LP-BM5 leukaemia virus (MuLV). LP-BM5 MuLV-infected mice characteristically develop hypergammaglobulinaemia, splenomegaly, lymphadenopathy, T-cell functional deficiency, B-cell dysfunction, and, in the later stages, neurological signs including paralysis as well as susceptibility to opportunistic infections (Liang et al., 1996). The similarities between murine AIDS and human AIDS are striking, with alterations in protein expression in a mouse model of AIDS in the presence or absence of chronic alcohol consumption.

Conclusions: These results suggest that chronic alcohol consumption may exacerbate the effects of viral infection on the heart by lowering the stress response leading to de-protection and further cytotoxic effects.

MATERIALS AND METHODS

Animals, infection and experimental groups
Female C57BL/6 mice were obtained from Charles River Laboratories Inc. (Wilmington, DE, USA). They were housed four mice per cage and kept in the animal facility of the Arizona Health Sciences Center. Water and diet were freely available (Harlan Diet 7002, Teklad, Madison, WI, USA). Food intake, fluid intake and body weight were measured every 2 days. The LP-BM5 murine leukaemia retrovirus mixture was administered intraperitoneally to mice in 0.1 ml minimal essential medium (MEM) with an esotropic titre of 4.5 log_{10} plaque forming units per 10^{-3}/l, which induces disease as previously described (Wang and Watson, 1994; Liang et al., 1996). Uninfected mice were injected with 0.1 ml of MEM and used as controls. All mice were 7 months old at the onset of treatment. Mice were randomly placed into four experimental groups of six mice each as follows: (1) uninfected; (2) uninfected, ethanol-treated; (3) LP-BM5 infected; (4) LP-BM5 infected, ethanol-treated. Ethanol-consuming mice received 2% sucrose (w/v) added to the drinking water to increase palatability. Non-ethanol fed controls received 2% sucrose in their drinking water. The ethanol concentration was increased in increments of 10%, at 1 week intervals, from 10% to a final concentration of 40% (v/v) in the water. Administration of ethanol was begun 2 weeks after LP-BM5 infection and the...
infection period was 18 weeks for all groups. Hearts were taken from all animals from each group, rapidly frozen in liquid nitrogen and stored at −70°C until proteins were extracted.

Preparation of tissue samples

All tissue samples were stored in liquid nitrogen prior to processing. The frozen tissue specimens (typically 0.2 g) were ground to a fine powder under liquid nitrogen using a pestle and mortar. The resulting powder was collected into 1.5 ml microcentrifuge tubes and homogenized using a handheld homogenizer for 1 min in 1 ml of lysis buffer, containing 9.5 M urea, 1% dithiothreitol (DTT), 2% CHAPS and 0.8% Pharmalyte pH 3–10 (Amersham Biosciences, Amersham, UK). We have found that this method of sample preparation is optimal for cardiac tissue and that reagents such as thiourea, alternative zwitterionic detergents and reducing agents do not give improved solubility or better separation of heart proteins by two-dimensional polyacrylamide gel electrophoresis (2-DE) (M. J. Dunn and J. Weekes, unpublished data). After vortexing for 30 s, samples were centrifuged at 15 000 r.p.m. (20 000 g) for 1 h and the resulting supernatants were collected. Total homogenate protein concentration was measured in duplicate using a modification of the method described by Bradford (1976). Briefly, the BSA used for preparation of standard curves and the protein samples to be measured were made up to 10 μl with lysis buffer prior to the addition of 10 μl of 0.1 M HCl and 80 μl of H2O. Bradford reagent diluted 1 in 4 with H2O was added to a final volume of 3.5 ml and the protein concentration was then determined spectrophotometrically.

Two-dimensional polyacrylamide gel electrophoresis

First-dimension isoelectric focusing (IEF) was performed using 18 cm immobilized pH gradient (IPG) strips (Amer sham Biosciences), with pH ranges 4–7 L (linear) and 3–10 NL (non-linear), using an in-gel rehydration method. The samples were diluted with rehydration solution containing 8 M urea, 0.5% CHAPS, 0.2% DTT and 0.2% Pharmalyte pH 3–10 prior to rehydration overnight in a re-swelling tray. For analytical gels, total protein loaded was 100 μg in a total volume of 450 μl, and for preparative gels, 400 μg in a total volume of 450 μl. The strips were focused at 0.05 mA/IPG strip for 60 kVh at 20°C. After IEF, the strips were equilibrated in 1.5 M Tris pH 8.8 buffer containing 6 M urea, 30% glycerol, 2% SDS and 0.01% bromophenol blue, with the addition of 1% DTT for 15 min, followed by the same buffer with the addition of 4.8% iodoacetamide for 15 min (Weekes et al., 1999). Second-dimension SDS–PAGE was performed using 22 cm 12% T, 2.6% C separating gels without a stacking gel, using a Hoefer DALT system. The separation was carried out overnight at 20 mA/gel at 8°C and was stopped just as the dye front left the bottom of the gels.

Protein visualization, densitometry and computer analysis

Analytical gels were fixed and stained using the Daiichi 2-D silver staining kit (Insight Biotechnology, Wembley, UK). Micro-preparative gels were stained using a modified version of the PlusOne silver staining kit (Amer sham Biosciences) that is compatible with mass spectrometry (Yan et al., 2000). All silver-stained gels were scanned at 100 μm resolution using a Molecular Dynamics Personal SI laser densitometer. Gels were analysed using Melanie II image analysis software (BioRad, Hemel Hempstead, UK) running on a Sun Sparc Ultra I workstation. After detection of spots, the gels were aligned, landmarked and matched. Gels were then placed into the appropriate experimental classes and differential analysis was performed. All spots that differed between the classes by 50% or more and passed a Student’s t-test (P < 0.05) were accepted as being significant. All gel spots detected as significantly different between the groups were then highlighted and checked manually to eliminate any artefactual differences due to gel pattern distortions, abnormal silver staining and inappropriately matched or badly detected spots. For simplicity, the optical density values for each spot were averaged across each group.

Peptide mass finger-printing by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

Protein spots were excised from preparative-scale 2-DE gels stained with modified PlusOne silver stain, cut into 1 mm cubes, destained by washing in 20 μl aliquots of 50 mM ammonium bicarbonate in 30% (v/v) acetonitrile for 1 h repeatedly until colourless. The samples were then dried in a centrifugal evaporator and treated as described by Yan et al. (2000). Modified (methylated) porcine trypsin (Promega, Southampton, UK) was prepared as a stock solution in water (0.1 μg/μl). For digestion, 4 μl of trypsin solution were added to 21 μl of Tris buffer (5 mM pH 8.8, prepared fresh for each use) and added to the gel pieces before incubation overnight at room temperature. Digestion was stopped by addition of 15 μl of 50% acetonitrile, 0.1% TFA. MALDI-MS was performed using a TofSpec 2E mass spectrometer (Micromass, Manchester, UK) operated in the positive ion reflection mode at 20 kV accelerating voltage with ‘time-lag focusing’ enabled. The matrix was α-cyano-hydroxy-cinnamic acid (4 mg/ml) as described by Yan et al. (2000). Peptide mass maps were searched against SWISS-PROT/TrEMBL release 35, using Protein Probe (Micromass), or against a non-redundant database maintained by the National Center for Biotechnology Information (NCBI) using the Mascot search engine.

RESULTS

Protein changes in murine heart

Figure 1 shows the expression pattern of proteins from normal uninfected murine heart separated by 2-DE using a linear IEF pH gradient of pH 4–7 L. On average, a total of 1125 protein spots was observed in uninfected heart patterns. There was no significant difference in the total number of protein spots between any of the experimental groups. The Melanie II quantitative 2-D gel analysis software allowed us to compare the patterns of protein expression from each experimental group in a pair-wise fashion. The following comparisons were carried out:

(1) When the 2-DE patterns of cardiac proteins from the uninfected mice with and without ethanol feeding were compared, a group of 40 protein spots were seen to be differentially expressed between ethanol-treated and -untreated hearts. Of this group, the majority of spots (34) were of decreased abundance in hearts of ethanol-treated animals. Six protein spots were of increased abundance in ethanol-treated tissue. This comparison indicates spots that were altered due to ethanol treatment alone.
(2) Comparison of the cardiac proteins from uninfected and infected, ethanol-treated mice indicated spots altered due to infection or ethanol or a combined effect of ethanol and infection — 53 proteins were reduced and 15 were increased in the infected, ethanol-treated group.

(3) Comparing cardiac protein profiles of the uninfected and infected groups detected spots altered due to infection. Any spots that were detected in this comparison and also appeared altered in comparisons (1) and (2) above were subtracted from this group, leaving the proteins that were altered due to infection alone. A total of 15 protein spots were reduced in expression and nine were increased.

Of the 24 spots altered due to infection alone, eight were altered by ethanol treatment. For six of these spots, the effect of ethanol was to bring these altered proteins back towards the levels seen in the uninfected hearts. Alterations of two proteins were exacerbated by ethanol. Examples of such spots are shown in the histogram plots from quantitative computer analysis in Fig. 2. An example of a region of the 2-D gel protein profile showing a protein spot that is modulated by viral infection and ethanol consumption is shown in Fig. 3.

**Protein identification**

In order to identify the proteins of interest, we performed micro-preparative 2-DE using extracts from control female C57BL/6 mice obtained in the Heart Science Centre at Harefield Hospital. Tissue from control mice of the same strain was used for protein identification, as there was insufficient tissue from the experimental animals for micro-preparative 2-DE (400 μg protein load). Spots matching those proteins of interest from analytical gels were excised, de-stained, subjected to tryptic digestion and the resulting peptide mass profiles were analysed using MALDI-TOF (time of flight) mass-spectrometry. Using this technique, we have positively identified two of the eight proteins that are altered by ethanol as the 27 kDa heat-shock protein (HSP27) and mitochondrial long-chain acyl-CoA thioesterase 1. Unfortunately, identification of the six remaining spots that were altered by ethanol was not successful using the above technique. This was due to the low abundance of these proteins and the difficulty of locating these spots on the micro-preparative 2-D gels.

**DISCUSSION**

The murine model of AIDS induced by LP-BM5 leukaemia virus displays a number of features of human AIDS (Liang et al., 1996), and has been a useful tool for the investigation of retrovirus-induced immunodeficiency and the effects of ethanol as an accelerating cofactor (Fitzpatrick et al., 1995).
Fig. 2. Histogram plots showing results from quantitative computer analysis of 2-D gel protein profiles.

(A) Examples of protein changes due to viral infection alone (spots 629, 763, 771, 876); (B) examples of protein changes due to viral infection alone, alcohol consumption alone, or both (spots 482, 536); (C) examples of protein changes due to viral infection and that are further altered by alcohol consumption (spots 431, 455, 736, 867, 883, 899). Virus + (dark bars), virus – (light bars).
This study represents the first proteomic analysis of global protein alterations in the murine model of AIDS induced by LP-BM5 leukaemia virus. Using a proteomic approach, we have demonstrated that the expression of a number of cardiac proteins is significantly altered upon viral infection. It is also apparent from this investigation that the expression of a number of proteins is specifically altered due to the effect of ethanol feeding in the absence of viral infection. Long-term heavy consumption of alcohol can lead to alcoholic cardiomyopathy with loss of contractile function and enlargement of the heart. Ethanol also has direct toxic effects on the heart (Davidson, 1989) and has previously been shown to further suppress resistance to opportunistic pathogens and accentuate retroviral immune dysfunction in murine AIDS (Watson, 1992; Liang et al., 1996; Lee et al., 1999). We hypothesized that the cardiotoxic effects of alcohol would accentuate those caused by retroviral infection of the heart. Using a proteomic approach, we have previously examined alterations in global protein expression in the human (Corbett et al., 1998), canine (Heinke et al., 1998, 1999) and bovine (Weekes et al., 1999) heart in dilated cardiomyopathy and heart failure. We have also investigated alterations in cardiac proteins following chronic ethanol consumption in rats, where proteomic analysis demonstrated significant changes in the relative amounts of various proteins including heat-shock protein (HSP) 60, HSP70 and desmin (Patel et al., 1997). In the present study, we have applied this proteomic approach to investigate alterations in protein expression brought about by LP-BM5 leukaemia virus in the murine heart. In addition, we determined the effect of ethanol consumption upon cardiac protein expression in the LP-BM5 leukaemia virus-infected mice.

As might be expected, the comparison of the uninfected and infected, ethanol-treated mice (which indicated spots altered due to infection or ethanol or a combined effect of ethanol and infection) produced the largest number of protein expression changes — 53 proteins were reduced and 15 were increased in the infected, ethanol-treated group. In a number of cases, the effect of infection mirrored that of alcohol. In most cases, the effect of the combination of alcohol and infection did not increase or decrease the effect of either treatment in isolation. Such examples are spots 536 and 482 shown in Fig. 2. It is not clear why this should be the case, but it is possible that these proteins may be part of the stress response, which may be activated in a similar fashion by both infection and alcohol feeding.

It is interesting to note that, of the 24 proteins shown to be altered in expression in the heart due to LP-BM5 infection alone, eight appeared to be further altered by ethanol feeding. However, of these eight proteins, the majority (six) were ameliorated by ethanol and for only two was the effect of infection exacerbated by ethanol. This may appear to indicate that alcohol could produce a level of cardiac protection from the virus, which would perhaps contradict the tenet that ethanol consumption aggravates the effects of AIDS. However, if these proteins represent part of a protective response to viral infection it is possible that the effect of alcohol consumption may lead to a de-protection of the cardiac myocyte and a consequent aggravation of the effects of infection. Indeed, one of the proteins altered in response to ethanol feeding was
identified as the 27 kDa HSP27. It is possible, therefore, that ethanol consumption may accentuate the effects of retroviral infection in the heart by down-regulating the antiviral response and thereby compromising the ability of the cardiac myocyte to protect itself against the effect of viral damage. The identification of the remaining differentially expressed proteins will contribute to the understanding of the biochemical alterations in murine heart after LP-BM5 infection and the role of ethanol in the disease.

Acknowledgements — We are grateful to Robin Wait (Kennedy Institute of Rheumatology, Imperial College School of Medicine, London) for carrying out the mass spectrometric analysis. This research was funded by NIH grant HL63667 (R.R.W.). M.J.D. and J.W. thank the British Heart Foundation for financial support of their work.

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