ANTIOXIDANT ENZYMES AS CANDIDATE GENES FOR DISEASE RESISTANCE IN SHEEP FACIAL ECZEMA

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SUMMARY

Facial eczema disease (FE) in ruminants is caused by a mycotoxin, named sporidesmin, which causes liver damage. Sporidesmin toxicity is caused by its ability to generate free radicals which disrupt cellular processes and structures, resulting in cell injury and death. We analysed a number of antioxidant enzymes involved in free radical detoxication as candidate genes for FE resistance. The genes were first mapped by genetic linkage using RFLP markers. The closest-linked informative microsatellite markers were then used for linkage studies in four FE resource families which were designed to show segregation in resistance/susceptibility to the disease. The results to date indicated that CuZn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), glutathione peroxidase (GPX1) and glutathione reductase (GSR) are not involved in FE resistance/susceptibility. We also ruled out a possible involvement of the serum proteins ceruloplasmin (CP) and transferrin (TF) in these families.

Keywords: facial eczema, mycotoxin, antioxidant enzymes, free radicals, genetic markers, QTL.

INTRODUCTION

The estimated annual cost of facial eczema disease (FE) is $63 million for the sheep and $5-20 million for the cattle industries (Garthwaite 1995). Facial eczema is the clinical symptom of this liver disease caused by the mycotoxin sporidesmin. The source of the sporidesmin is *Pithomyces chartarum*, a saprophytic fungus endemic in northern New Zealand pastures. During autumn, when the weather is warm and humid, the fungus proliferates and forms spores which contain the hepatotoxin. Animals grazing on such spore-infested pastures are likely to ingest the toxin which causes liver damage in susceptible animals, leading to cholestasis. Phylloerythrin is a break-down product of plant chlorophyll by microbes in the gut. In healthy animals assimilated phylloerythrin is normally conjugated and excreted in the bile. FE-affected animals with dysfunctional livers accumulate phylloerythrin in their circulatory system. Phylloerythrin is a photodynamic pigment which becomes reactive upon absorption of ultra-violet light and causes local lesions, particularly around the exposed facial tissues: hence the name facial eczema.

The sporidesmin molecule contains an epidithiopiperazinedione moiety. Within liver cells, cyclic reduction-oxidation of the disulphide group produces superoxide radical (Munday 1989),

which initiates a cascade production of other toxic free radical species. Additional to free radicals already generated from normal cellular processes, it is suggested that the influx of free radicals from sporidesmin-induced production overwhelms the cellular detoxication systems, leading to cell death and liver dysfunction.

It is known that there are heritable differences among sheep in their resistance/susceptibility to the harmful effects of sporidesmin (Morris *et al.* 1995). In view of the involvement of free radicals in this process, such heritable differences could reflect differences in the ability of the animals to produce or detoxify such radicals. Using the candidate gene approach to isolate disease genes to FE, we have analysed four antioxidant enzymes involved in cellular free-radical production/removal and two serum proteins for their possible involvement in the disease traits.

MATERIALS AND METHODS

Genomic DNAs were prepared from heparinised blood, and enzyme digestions were done as recommended by the suppliers. 0.8% or 1.0% agarose-gels were routinely used to resolve the DNA bands for Southern blottings. The probes used were cDNA clones of either human (SOD2, GSR, CP) or cattle (SOD1, GPX1) origin, and were [α-^{32}P]dCTP-labelled using the random primers method. Southern hybridizations and washings were done at 60°C, and final stringency of washing was in 1xSSC-0.1%SDS (Sambrook *et al.* 1989). Primers were kinased with [γ-^{33}P]ATP, and the touch-down PCR conditions were used in genotyping with microsatellite markers (Crawford *et al.* 1995). Exposure time of autoradiography ranged from one to seven days.

Four sires were obtained from reciprocal crosses between FE resistant and susceptible selection-line animals (Morris *et al.* 1995). The sires were each mated to about 150 unselected ewes to generate the four half-sib resource families, with ~150 offspring per family. The offspring were dosed with sporidesmin and their serum gamma-glutamyl transferase (GGT) levels were measured. The level of serum GGT is related proportionally to the extent of liver damage (Towers & Stratton 1978), and was used to quantify the degree of susceptibility/resistance of an animal to sporidesmin. Only the 25 most resistant and 25 most susceptible animals from each family were analysed. Quantitative trait locus analysis was performed by comparing groups inheriting alternative sire alleles using least square methods. A contingency table analysis comparing independence of sire allele and susceptibility group was also conducted.

RESULTS AND DISCUSSION

We first identified RFLP markers for the genes by screening the respective cDNA probes against six unrelated sheep, digested with 16 restriction enzymes: *Apa*I, *Bam*HI, *Bgl*II, *Bst*EII, *Bst*XI, *Eco*RI, *Eco*RV, *Hind*III, *Hinf*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, *Sca*I, *Taq*I and *Xba*I. The genes were then placed on the sheep genetic linkage map by genotyping the three-generation AgResearch International Mapping Flocks animals (Crawford *et al.* 1995) using the identified RFLP markers (Table 1). TF was mapped half-way between the CP locus and the BM1824

marker. The closest-linked microsatellite markers, which showed heterozygosity in the sires, were used for linkage studies in the resource families which showed segregation of the disease traits (Table 2). The results showed no segregation of the markers with disease traits. Our analysis took into account only the sire's genetic contribution to the half-sibs' disease phenotype: this approach might not detect resistance/susceptibility genes which are recessive. Also, though antioxidant enzymes were good candidate genes, they might not be the genes responsible for the disease traits present in our resource families (Morris *et al.* 1988).

Table 1. Candidate genes, their RFLP markers and chromosomal assigments

Candidate gene	RFLP marker		Sheep chromosome
	Restriction enzyme	Allelic bands (Kb)	
CP (ceruloplasmin)	*Eco* RI	4.2 & 2.7	1
SOD1 (CuZn-superoxide dismutase)	*Msp* I	8.0 & 7.4	1
SOD2 (Mn-superoxide dismutase)	*Apa* I	10.5 & 7.8	8
GPX1 (glutathione peroxidase)	*Taq* I	3.4 & 0.6	19
GSR (glutathione reductase)	*Pst* I	4.1 & 1.0	26

Table 2. Two-point linkage analysis of candidate genes with informative microsatellite markers, and the latter's tests of association with FE resistance in resource families

Candidate gene	Linkage[A]			Test in resource families	
	Markers	Theta	Lod score	Family tested[B]	Result[C]
CP	BM1824	0.12	8.9	2, 3 & 4	NS
	MAF109	0.14	6.7	1, 2, 3 & 4	NS
	BM864	0.13	6.3	2 & 4	NS
	TF	0.19	3.5		
SOD1	BM6438	0.01	20.2	1 & 2	NS
	MAF64	0.13	9.9	1 & 4	NS
	RM65	0.18	5.1	4	NS
SOD2	BM3215	0.03	15.8	1, 2 & 3	NS
	BM4208	0.06	8.3	2, 3 & 4	NS
GPX1	OarFCB304	0.03	12.4	2 & 3	NS
	OarCP88	0	6.6	2 & 4	NS
GSR	OarJMP58	0.09	9.6	3 & 4	NS

[A]Analysis using the TWOPOINT option of CRI-MAP.

[B]The four half-sib resource families are designated family 1 to 4.
[C]Quantitative trait locus analysis with P>0.05: NS denotes "not significant".

The study of enzymes involved in the liver detoxication processes is a different approach to selecting candidate genes. There are two major detoxication pathways of xenobiotics: oxidation-reduction and conjugation/hydrolysis. The first pathway introduces polar groups on an otherwise lipophilic xenobiotics, enabling the metabolites to be readily excreted in urine or bile; the enzymes responsible for this process include dehydrogenases, oxidases, mono-oxygenases and reductases. It should be noted that this redox detoxication route can inadvertently produce a more reactive, and hence more toxic, intermediate than the parent compound.

Detoxication via the conjugation and hydrolysis pathway involves conversion of a pharmacologically active xenobiotics into either an inactive derivative, a polar derivative which is readily excretable, or both. The candidate genes for conjugation reactions include enzymes involved in glucuronidation, the glutathione system, *N*-acetylation, methylation and sulfation. In the hydrolysis process, candidate genes are amidases, epoxide hydratase, esterases and imidase.

We have not yet begun analysing enzymes involved in the two detoxication pathways above. Work on the MDR genes, which are transmembrane P-glycoproteins involved in multidrug resistance, is in progress.

REFERENCES

Crawford, A.M., Dodds, K.G., Ede, A.J., Pierson, C.A., Montgomery, G.W., Garmonsway, H.G., Beattie, A.E., Davies,K., Maddox, J.F., Kappes, S.W., Stone, R.T., Nguyen, T.C., Penty, J.M., Lord, E.A., Broom, J.E., Buitkamp, J., Schwaiger, W., Epplen, J.T., Matthew, P., Matthews, M.E., Hulme, D.J., Beh, K.J., McGraw, R.A. and Beattie, C.W. (1995) *Genetics* **140**: 703-724.

Morris, C.A., Jordan, T.W., Loong, P.C., Lewis, M.H. and Towers, N.R. (1988) *N. Z. J. Agr. Res.* **31**: 301-305.

Morris, C.A., Towers, N.R., Wheeler, M. And Wesselink, C. (1995) *N. Z. J. Agr. Res.* **38**: 211-219.

Munday, R. (1989) *Free Radical Biology & Medicine* **7**: 659-673.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) "Molecular Cloning: A Laboratory Manual" 2nd ed. Cold Spring Harbor Laboratory Press, USA.

Towers, N.R. and Stratton, G.C. (1978) *N. Z. Veterinary Journal* **26**: 109-112.

Towers, N.R. (1995) In "Toxinology and Food Safety Research Report 1992-1995", p.1, editor L. Garthwaite, AgResearch Ruakura, Hamilton, New Zealand.