Toxicogenomics and Its Potential for Assessment of Pollutant Impact in Fish

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Slide 1 Good evening, thank you for the kind introduction, my name's Tim Williams and I'd like to tell you about the work of our research group at the University of Birmingham.

We are using toxicogenomics to study pollution of the marine environment by various classes of chemicals including xenoestrogens.

Slide 2 Why are we interested in monitoring the marine environment? Marine pollution adversely affects the fishing industry, recreation and tourism and decreases biodiversity. Therefore we need sensitive pollution indicators which provide an early warning of problems before extensive damage is caused. These will aid the management of the coastal environment, monitor the impact of industry, encourage ecologically sustainable industrial development and highlight problems associated with novel chemicals. Toxicogenomics is not at a stage where it can be usefully applied to regulatory programs, but we hope to use it to develop assays suitable for regulatory application in the future. First we must consider how organisms react to chemical contamination.

Slide 3 In general, as the severity of effect increases, due, for example, to an increase in concentration of a chemical, different responses are seen within the organism. For most toxicants there will be a level where there is no effect on the organism, subsequently a stress response may be seen, such as adaptive gene expression changes. At greater severity, potentially repairable damage is seen, this can lead to pathological changes in an individual and then on to serious population effects such as death of individuals or an inability to reproduce. If these changes are detected, they may be used as biomarkers of exposure and effect. These are especially useful at low levels of effect where changes are difficult to find by studying whole populations.

Slide 4 So what do we mean by a biomarker? One definition is that it's a substance, structure or process that can be measured in an organism or its products, which is indicative or predictive of exposure or damage. Typical molecular biomarkers used for environmental monitoring include vitellogenin, which is induced by xenoestrogens, cytochrome P450 1A, induced by polycyclic aromatic hydrocarbons and dioxins, and metallothionein, induced by heavy metals. These inductions may be detected by increases in mRNA or protein levels, or, in the case of cytochrome P450, by enzyme activity. However there are a number of complexities when animals are sampled from the environment. These include the effects of mixtures of chemicals, environmental changes such as temperature and diet, the age of the organisms and stage of the breeding cycle. Therefore individual biomarkers may provide misleading results. An improved approach would be to use a larger range of traditional and novel biomarkers producing 'fingerprints' of change reproducibly associated with specific toxic responses. Due to the crosstalk between systems of gene regulation we believe it is necessary to study xenoestrogen contamination in the

context of the complex chemical mixture, including polycyclic aromatic hydrocarbons and metals, found at polluted environmental sites.

Slide 5 Toxicogenomics may be defined as the combination of information from genomics and bioinformatics in order to identify and characterise mechanisms of toxicity. The technique requires the analysis of gene expression on a large scale, key technologies include subtractive hybridisation, differential display RT-PCR and Serial Analysis of Gene Expression. Today I'll mainly be talking about DNA microarraying, which allows the expression of thousands of genes to be assessed simultaneously. However, one first needs to isolate the genes. At present, the only fish genome sequencing projects are for Fugu and Zebrafish, but these organisms are not suitable for monitoring in European seas as they are not indigenous. We aim to apply high throughput genomic technologies to a species which is not currently a genetic model. Using this information we aim to find gene expression patterns characteristic of specific pollutant responses.

Slide 6 The organism we study is the European flounder *Platichthys flesus*. This species is quite similar to the Japanese flounder or Hirame (*Paralichthys olivaceus*), but grows to only about one third of the size. It is common in Western European coastal waters and feeds on invertebrates found in estuarine sediment where it is often highly exposed to anthropogenic pollutants. It is a key species in the British national marine monitoring program and has been the subject of much previous research by our group and others, so we already had cDNA and genomic libraries. Our fish samples were kindly provided by Dr. Lyons from the Centre for the Environment, Fisheries and Aquaculture (CEFAS).

Slide 7 Our laboratory is located in Birmingham in central England and CEFAS is on the east coast. These are our two sampling sites, the Alde estuary in Suffolk, which is an agricultural area, and the Tyne estuary at the heavily industrialised city of Newcastle.

Slide 8 The Tyne estuary is affected by many pollutants. It is exposed to 10,000 times more industrial and domestic effluent than our reference site, the Alde. Contamination by polycyclic aromatic hydrocarbons has resulted in a high level of bile metabolites and DNA adducts, indicative of genotoxicity. There is also substantial endocrine disrupter contamination. As can be seen from this table, the majority of estradiol is found in sediment solids, with which the flounder are closely in contact.

Slide 9 Part of our research in aquatic toxicology is based on the cloning of known stress-responsive genes from the flounder. We have used these for reporter gene assays and quantitative competitive RT-PCR assays and these techniques could be applied to detection of xenoestrogens. However, I'm going to concentrate on our arraying studies.

Slide 10 We have completed an initial array project, and, as we are using a non-model organism, needed to clone the genes ourselves. We have used a mini-array focussed on specific stress responsive genes to compare flounder from the Tyne and Alde. Our current work involves the isolation of a large

number of gene targets, which we are obtaining by subtractive hybridisation, and random isolation of up to 10,000 expressed sequence tags (ESTs).

Slide 11 Our cloning strategy for the initial focussed array project was to design degenerate primers based on the sequences of homologous genes from different organisms. We then PCR-amplified fragments of around 400 base pairs from flounder DNA and cloned and sequenced these.

Slide 12 This resulted in the cloning of fragments of genes from a range of classes, including those for biotransformation enzymes such as cytochrome P450s, oxidative stress-related proteins such as superoxide dismutase, heat shock proteins, oncogenes and others, for a total of 120 novel transcripts. The endocrine related genes included zona pellucida protein C and a putative membrane associated progesterone receptor. Vitellogenin was not isolated but has recently been cloned for flounder and we will include it in future arrays.

Side 13 We amplified these clones using vector primers and arrayed them onto Corning glass slides using a high precision robot. cDNA was synthesised from our test and control fish and labelled with either the red fluorophore Cy5, or the green fluorophore, Cy3. The mixture of labelled cDNA was hybridised to the array and spot intensities measured using an Axon scanner. The ratio of red label to green label indicates the ratio of expression of each gene in the test and control samples.

Slide 14 This shows a scan of one of our arrays, most genes are not differentially expressed, so show up as yellow. Those that are differentially expressed show up as red or green.

Slide 15 This shows a typical set of results from the array, showing genes up-regulated in the Tyne in red and orange and the genes down-regulated in Tyne in green. I've noted some of these. Zona pellucida protein C (ZPC), NADP-menadione oxidoreductase (NMO), glucose-6-phosphate dehydrogenase (G6PDH), δ-aminolevulinic acid synthase (δ-ALAS) and metallothionein (MTT) are up-regulated, as well as some unidentified transcripts. γ-fibrinogen (γFIB) and α-2HS glycoprotein (α2HSGP) are amongst the down-regulated transcripts with respect to pollution.

Slide 16 Here are some examples of the differentially regulated genes. Those up-regulated in polluted conditions are ZPC, which I'll discuss further in the next slide, NMO, which may be induced by hydrocarbon pollutants, δ-aminolevulinic acid synthase, which is the key enzyme of heme synthesis and metallothionein, the classical indicator of heavy metal pollution. Maybe the most interesting are some gene fragments which remain unidentified. These could indicate novel responses to pollution. The genes down-regulated in Tyne include α-2HS-glycoprotein, which is known to be down-regulated during the acute phase response, and gamma fibrinogen which may be affected by a range of pollutants including xenoestrogens.

Slide 17 The most relevant of these genes for the study of endocrine disrupters is zona pellucida protein C, also called ZP3 or ZRP. It is a member of a multigene family of egg envelope proteins conserved throughout vertebrates, but is unlikely to have the same role in sperm recognition as the mammalian protein due to the different structure of the teleost oocyte. These proteins have been proposed as sensitive biomarkers of estrogenic contamination by a number of research groups. In fish there appear to have been a number of duplications of this gene and the ZP proteins are expressed in liver or ovary in different species. As our gene fragment contained a highly conserved region, it is likely that we are seeing the differential regulation of a group of similar genes rather than one unique transcript. In collaboration with Dr. Hughes from Birmingham, we are investigating the various transcripts which may be produced in flounder liver and ovary and plan to design unique probes for each specific transcript. This shows how arraying can draw attention to specific genes which can be studied in greater detail and may be applied to monitoring.

Slide 18 Our current work is a considerable expansion of the arraying project and links the genomics approach to proteomics. This is a European Union funded project called GENIPOL; Genomic tools for bio-monitoring of pollutant coastal impact. The consortium is made up of groups from Birmingham, Stirling in Scotland, Odense in Denmark and Haifa in Israel. We are combining the specific genes isolated previously with genes from subtractive hybridization and are randomly isolating a large number of expressed sequence tags from flounder cDNA. These clones will be used for arraying to identify those genes which are differentially expressed.

We believe it is essential to distinguish responses which are due to pollutants and those which are non-specific. Clones useful as biomarkers will be used for real time PCR assays and to produce a mini-array with which we aim to test flounder from a number of sampling sites around Europe. Parallel to this approach, two-dimensional polyacrylamide gels will be used to identify differentially regulated proteins. We aim to use those proteins to raise antibodies for enzyme-linked immunosorbent assays (ELISA) to create convenient assays suitable for routine monitoring. The results of the flounder studies will be applied to the Mediterranean fish, Striped Sea Bream (*Lithognathus mormyrus*) which will allow us to monitor sites around the entire coast of Europe. Therefore we have completed our pilot project and are moving on to extend the scale and scope of our studies. Perhaps the greatest challenge of this project will be the integration of large amounts of data from these varying sources.

Slide 19 I'd like to thank Kazuhiko Adachi, Tohru Inoue and Kathleen Cameron for inviting me to speak here, and the Japanese Ministry of the Environment and UK-Japan Collaboration on Endocrine Disrupters for facilitating this visit.

I'd also like to acknowledge Brett Lyons for the fish samples, Antony Jones and Steve Minchin for Genomics support, Karl Gensberg, Tanya Franklin and Jae-Seong Lee for useful clones and our Professor, Kevin Chipman for organising these projects.

Q&A

Blumberg: Thank you very much, Prof. Williams. We have time for a few questions.

Q: Are these hydrocarbon receptors down-regulated in most of the cases?

Williams: We have included the aryl hydrocarbon receptor in this array. We have not seen a great response from it, yet. I think it is slightly up-regulated, but not below the two-fold cutoff which is usual for significance in array studies.

Daston: Just a comment. The up-regulation of metallothionein in your stressed fish may, as you suggest, be an indication of heavy metal exposure. It might also, though, be an indication of a more generalized acute phase response. At least in mammals, metallothionein induction is fairly common as an acute phase reactant, and since you saw the down-regulation of the serum acute phase reactant, it may be the case. It would be I think interesting to know whether there are other acute phase reactants that you have measured, and do you think that this could be useful as a suite of genes that you could use to differentiate specific kinds of toxicity from generalized stress?

Williams: Yes, I take your point about metallothionein. I think, in the case of most of these genes, there is more than one possible explanation for their differential regulation. Therefore, we hope to dissect the possibilities by using single exposures in tech studies, in laboratory studies, with these organisms. We are using a wide range of different treatments and hope to cluster the responses from the array, which should hopefully start to give us the answers to questions like these.

Blumberg: Any other questions?

Q: Did you check the protein induction like metallothionein or…

Williams: No, this will go ahead in the next section of the project, which we'll be studying ptroteomics.

Q: And did you check the water contamination like chemicals, metals or solvents or something like that?

Williams: We will be using a wide range of compounds including metals.

Q: Especially for organotin compounds, I want to know the analysis.

Williams: Organotin, TBT, yes, we will be including TBT in this study.

Blumberg: OK, thank you very much Prof. Williams.