BIOMARKERS, 1999, VOL. 4, NO. 6, 492–496

DNA damage as a bivalve biomarker

SCOTT A. STEINERT

Marine Sciences Department, Computer Sciences Corporation, 4045 Hancock Street, San Diego, CA 92110, USA. e-mail: steinert@spawar.navy.mil

Bivalves have been used in numerous environmental assessment studies, chiefly because they are sessile deposit or suspension feeding organisms found in or near sites of environmental concern, and they can be easily collected, sorted and deployed at sites of interest. Monitoring studies utilizing bivalves currently rely on the comparison of growth, survival, and contaminant bioaccumulation. Data gathered from 'reference' sites are compared with those of populations at assessment sites. These studies require extended periods of exposure, lasting weeks to months, and the use of a well defined population of test organisms of similar size, age, and condition. In many cases time and resources require researchers to restrict their sampling to the organisms on-hand at a particular site without the benefit of any reference data. Therefore more versatile and sensitive assessment methods are needed. Because effects at higher levels of organization such as growth, development, and survival are initiated at the molecular and cellular levels attempts have been made to identify useful biomarkers at these levels. The proposed advantages of molecular/cellular biomarkers are that they will respond to stress predictably and more rapidly, and will be indicative of the mechanisms of toxicity thereby yielding a rudimentary characterization of the contaminant(s) influencing them. In the following communication we will report on past and current developments in the monitoring of DNA damage as an environmental biomarker.

Keywords: bivalves, environmental contaminants, biomarkers, DNA damage.

Introduction

Maintaining DNA integrity is of paramount importance to all living things. For this reason living organisms process very efficient and intricate mechanisms for the protection of their genetic material. Significant stresses eventually result in the dysfunction of these mechanisms and an increase in observed DNA damage (Shugart *et al.* 1992). To date this laboratory has studied DNA damage in response to stress in cells from over three dozen plant and animal species using the Comet assay. DNA strand breaks are commonly detected by measuring the integrity of DNA after denaturation or unwinding under alkaline conditions. The Comet assay, also called the single-cell gel electrophoresis assay, has proven to be a rapid, versatile and easily utilized tool for collecting data on DNA strand breakage (Singh *et al.*, Rydberg and Johanssen, McKelvey-Martin *et al.* 1993). Sample preparation requires as few as 10000 cells. This method measures the electrophoretic migration of relaxed or fragmented DNA away from the nuclei of cells immobilized in agarose gel. The DNA is stained with a fluorescent nucleic acid stain and viewed using an epifluorescent microscope. The distance and/or amount of DNA migration from individual nuclei is indicative of the number of strand breaks. The microscopic determination of DNA migration can be collected either by eye using an ocular micrometer or with the use of image analysis software. Image analysis systems are comprised of a CCD camera attached to an epifluorescent microscope and software designed specifically to capture and analyse images of fluorescently-stained nuclei. Using such a system it is possible to measure the fluorescence intensity and distribution of DNA in and away from the nucleus (Singh 1996).

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
http://www.tandf.co.uk/JNLS/bmk.htm
http://www.taylorandfrancis.com/JNLS/bmk.htm

Past and current studies

To demonstrate the overall utility and versatility of this technique for the determination of organismal health most studies have been designed to demonstrate a link between the levels of DNA damage and significant effects on higher order endpoints such as mortality, growth, and development (Steinert 1998a, b, Lee *et al.* 1999). In order to investigate the utility of this approach the test organisms in many of these studies have been field-deployed bivalves collected from sites with low contaminant impact. The use of these sessile organisms allows the simple collection, sorting and deployment of organisms from well defined reference populations and the simultaneous collection of bioaccumulation, and whole organism level data in addition to cellular biomarker data.

A great deal of relevant information can be extracted from the analysis of Comet slide preparations. At this laboratory the yield of information from Comet assay studies has continuously increased and evolved with each study. Sample collection and analysis is fairly simple and straightforward. Cell samples are collected from bivalves by drilling a small notch in the shell of each organism above the posterior adductor muscle to allow the introduction of a syringe needle, and haemolymph (50–100 μl) is withdrawn from the posterior adductor muscle with a 26 gauge needle and 0·5 ml syringe. In organisms sampled just prior to spawning, gonadal tissue surrounds the posterior adductor muscle, and sperm and eggs may infiltrate haemolymph samples. Scoring by eye requires measuring the diameter of the nucleus and the distance of DNA migration from the nucleus. In initial studies significant increases in the frequency of cells with damaged DNA were found in mussels with greater exposure to contaminants (Steinert *et al.* 1998a). It was found that sperm, egg and somatic cells could be distinguished from one another on the basis of nuclear diameter and, in the case of eggs, by their unique crescent appearance.

On a Comet slide sperm are identifiable as abundant small diameter (≤12 μm) nuclei. Infiltration by eggs is not as prevalent as with sperm, possibly because of their large size, >35 μm. We suspect that large fully developed eggs create a large impression or void in the agarose. Consequently the egg's nuclear material, free-floating in this chamber after lysis, freely migrates during electrophoresis until it contacts the curved agarose wall of the original impression, where it is immobilized along this curvature appearing as a crescent of stained DNA. Because of the difficulty measuring these crescents, eggs are not scored.

By distinguishing between sperm and somatic cell nuclei, variability was reduced and it was found that observed DNA damage was consistently higher in sperm cells than in somatic cells, as seen in figure 1 (Steinert *et al.* 1998a). In laboratory exposure experiments DNA repair has been observed in somatic cells but never in sperm (Steinert 1996). This lack of DNA repair has been reported for mammalian sperm as well (Genescà *et al.* 1992). Because of this reduced repair capacity sperm cells represent a less malleable DNA damage record than do somatic cells, therefore resulting in a steeper response curve. In addition, it is likely that a large difference between somatic and germ cell DNA damage may be indicative of highly induced DNA repair activity in somatic cells.

Significant differences of this type have been most notable in mussels exposed to PAHs (Steinert *et al.* 1998a). Whereas in mussels exposed to copper, somatic damage was closer to germ cell damage levels presumably because a higher frequency of these cells were becoming apoptotic and incapable of repair (Steinert

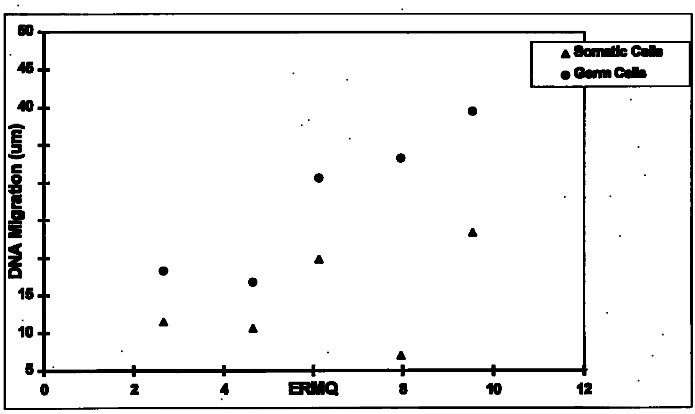

Figure 1. Plot of DNA damage measured in germ cells and somatic cells in relation to effects-based sediment contaminant concentrations at sites in San Diego Bay where the sampled mussels were deployed for 32 days. Reproduced from Steinert *et al.* 1998a).

et al. 1998a). We have shown that in *Mytilus galloprovincialis*, observed increases in DNA damage were partially a consequences of the induction of apoptosis (Steinert 1996). Apoptosis is characteristically an orderly sequence of events that ultimately leads to the complete disassembly of the cell (Buja 1993). Apoptosis results in the fragmentation of DNA and can appear as DNA damage resulting from genotoxicity when cellular DNA extracts are examined. Using the Comet assay rather distinctive micronuclei are found to be associated with apoptotic cells making it possible to estimate the frequency of cells undergoing apoptosis. Large increases in apoptotic frequencies have been observed in cells from mussels at contaminated sites in apparent response to copper (Steinert *et al.* 1998a) and in response to copper in laboratory studies (Steinert 1996).

It has therefore been demonstrated that without the aid of an image analysis system a great deal of information can be gathered using the Comet assay. The downside is that data collection is rather time consuming and is more vulnerable to human error. Using an image analysis system one can more accurately determine the relative concentration of DNA by its staining intensity and in so doing distinguish different cell types by their DNA content, and the tedious nature of the analysis is vastly reduced. Image analysis allows more precise identification of germ cells (sperm) and somatic cells, and the distinction of somatic cells at different stages of the cell cycle. Therefore, cells with vastly different sensitivities to various stressors and different capabilities for handling DNA damage can be distinguished from one another rapidly, more accurately, and less subjectively.

In addition to the appearance and staining characteristics of different cells far more information can be gleaned by adjusting lysis and electrophoresis conditions and/or by incorporating enzyme or inhibitor treatments. The alkaline lysis and electrophoresis conditions used in the above studies are used for the detection of single-stranded DNA damage, whereas neutral pH conditions facilitate the detection of double-strand breaks (Singh 1996). Other researchers have incorporated chemical treatments to express specific types of DNA damage or, as in one method, to preserve strand damage at sites of DNA repair (Gedik *et al.* 1992). It has been demonstrated that contaminant exposure does lead to corresponding increases in DNA damage. However, the other side of the DNA

damage equation, DNA repair, has received little attention and may be just as important a measure of organismal health as the determination of DNA damage. As reported by Gedik *et al.* (1992), repair activity can be determined by treatment of cells with the excision repair ligation inhibitor aphidicolin. Aphidicolin inhibits the ligation stage of repair, resulting in an accumulation of gaps in the DNA of treated cells whereas repair has occurred. This results in higher levels of damage at higher rates of repair which are detectable using the Comet assay.

In recent years the Comet assay has been modified by the addition of nuclease digestion steps in order to introduce strand breaks at specific lesion sites. This approach has been pioneered by Collins *et al.* in a series of studies in which oxidative base damage was detected by the use of endonuclease III (Collins *et al.* 1997), as well as DNA modifications resulting from exposure to ultraviolet light (UV) through the use of T4 endonuclease V (Collins *et al.* 1993). These procedures are similar to the aphidicolin method but have the advantage that they are conducted on the already immobilized cell nuclei following lysis, and therefore do not require the use of living cells. Modifications of this type vastly expand the utility of this assay and are good examples of its versatility.

Along the same lines, this laboratory has recently used ultraviolet light treatments as a diagnostic tool to detect bioaccumulated photoactive agents, such as polycyclic aromatic hydrocarbons (PAHs), in mussel tissues. In one study bioaccumulation of PAHs in mussels deployed at a PAH-contaminated site was detected by UV exposure of mussel haemocytes (Steinert 1998b). Mussels, *Mytilus galloprovincialis*, were deployed at a PAH-contaminated and a reference site under light-exposed and shaded conditions. According to growth and background DNA damage measures, the PAH-exposed and shaded organisms were not obviously stressed. The matching group of mussels exposed to sunlight experienced significantly reduced growth and increased DNA damage. The presence of the bioaccumulated PAHs in the shaded mussels was detected by the induction of DNA damage following a 30 min UV exposure of haemocytes in the laboratory. Mussels at the reference site showed no damage increase. A similar result to UV treatment was found in a sediment deployment study using the infaunal mussel, *Musculista senhousia*. Of five stations, the two stations with significantly increased DNA damage following laboratory UV treatment were those at which significant elevation in PAH bioaccumulation was found (Steinert *et al.*). This treatment shows promise as a means to identify the presence of accumulated photoactive compounds, and demonstrates the significant toxic impact expressed as a result of PAH photoactivation.

The Comet assay has been found to yield essential information on the severity of damage, potential long-term effects, and contaminant class responsible for the observed DNA damage. The degree of strand breakage and the frequency and types of cells with significant levels of DNA damage is indicative of the severity of the stress. Germ cell damage may have significant downstream affects on the reproductive success of an individual organism or population. The effect of UV light on DNA damage levels is an indicator of the presence of photosensitive contaminants, such as PAHs. It is also possible to measure rates of DNA repair and identify apoptotic cells using this assay. Though DNA damage at first glance appears to be a general indicator of stress, there is evidence that specific cell treatments and data analysis methods can yield contaminant-specific information. Because of the simplicity of the assay, chronic effects can be distinguished from

transient effects through repeated sampling or through the correlation with bioaccumulation and other higher order measurements such as growth, reproduction, and development. These simple measures all made within the framework of the same assay, increase our understanding of the true impact various environmental stressors have on an organism or population and currently appears to be a very useful environmental assessment biomarker.

References

BUJA, L. M., EIGENBRODT, M. L. and EIGENBRODT, E. H. 1993, Apoptosis and necrosis: basic types and mechanisms of cell death. *Archives of Pathology and Laboratory Medicine*, **117**, 1208–1214.

COLLINS, A. R., DOBSON, V. L., DU, M., KENNEDY, G. and TINA, R. 1997, The comet assay: what can it really tell us? *Mutation Research*, **375**, 183–193.

COLLINS, A. R., DUTHIE, S. J. and DOBSON, V. L. 1993, Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, **14**, 1733–1735.

GEDIK, C. M., EWEN, S. W. B. and COLLINS, A. R. 1992, Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. *International Journal of Radiation Biology*, **62**, 313–320.

GENESCÀ, A., CABALLÌN, M. R., MIRÓ, R., BENET, J., GERMÀ, J. R. and EGOZCUE, J. 1992, Repair of human sperm chromosome aberrations in the hamster egg. *Human Genetics*, **89**, 181–186.

LEE, R. F., STEINERT, S. A., NAKAYAMA, K. and OSHIMA, Y. 1999, Use of DNA strand damage (Comet assay) and embryo development defects to assess contaminant exposure by blue crab (*Callinectes sapidus*) embryos. In *Environmental Toxicology and Risk Assessments*: 8th Volume, ASTM STP 1364, D. Henshel, ed. (American Society for Testing and Materials).

MCKELVEY-MARTIN, V. J., GREEN, M. H. L., SCHMEZER, P., POOL-ZOBEL, B. L., DE MÉO, M. P. and COLLINS, A. R. 1993, The single cell gel electrophoresis assay (comet assay): a European review. *Mutation Research*, **288**, 47–63.

RYDBERG, B. and JOHANSSEN, K. J, 1978, Estimation of DNA strand breaks in single mammalian cells. In *DNA Repair Mechanisms*, P. C. Hanawalt, E. C. Friedberg and C. F. Fox, eds (New York, Academic Press), pp. 465–468.

SHUGART, L., BICKMAN, J., JACKIM, E., MCMAHON, G., RIDLEY, W., STEIN, J. and STEINERT, S. A. 1992, DNA alterations. In *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*, R. J. Hugget, R. A. Kimerle, P. M. Mehrle, Jr, and H. L. Bergman, eds (Chelsea, MI: Lewis Publishers), pp. 125–153.

SINGH, N. P., MCCOY, M. T., TICE, R. R. and SCHNEIDER, E. L. 1988, A simple technique for the quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, **175**, 184–191.

SINGH, N. P. 1996, Microgel electrophoresis of DNA from individual cells: principles and methodology. In *Technologies for Detection of DNA Damage and Mutations*, G. P. Pfeifer, ed. (NY: Plenum Press), pp. 3–24.

STEINERT, S. A. 1996, Contribution of apoptosis to observed DNA damage in mussel cells. *Marine Environmental Research*, **42**, 253–259.

STEINERT, S. A., STREIB-MONTEE, R., LEATHER, J. M. and CHADWICK, D. B. 1998a, DNA damage in mussels at sites in San Diego Bay. *Mutation Research*, **399**, 65–85.

STEINERT, S. A., STREIB-MONTEE, R., and SASTRE, M. P. 1998b, Influence of sunlight on DNA damage in mussels exposed to polycyclic aromatic hydrocarbons. *Marine Research*, **46**, 355–358.

STEINERT, S. A., STREIB-MONTEE, R., LEATHER, J. M. and CHADWICK, D. B. *In situ* bioaccumulation and biomarker studies. In *Sediment Quality Characterization-Naval Station San Diego, Final Report*, SPAWAR Systems Center, San Diego, Technical Publications (in preparation).