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Regulation and Disregulation of Mammalian Nucleotide Excision Repair: a Pathway to Non-germline Breast Carcinogenesis†

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Abstract

Nucleotide excision repair (NER) is important as a modulator of disease, especially in constitutive deficiencies, such as the cancer predisposition syndrome Xeroderma pigmentosum. We have found profound variation of NER capacity among normal individuals, between cell-types and during carcinogenesis. NER is a repair system for many types of DNA damage, and therefore many types of genotoxic carcinogenic exposures, including ultraviolet light, products of organic combustion, metals, oxidative stress, etc. Since NER is intimately related to cellular metabolism, requiring components of both the DNA replicative and transcription machinery, it has a narrow range of functional viability. Thus, genes in the NER pathway are expressed at the low levels manifested by, for example, nuclear transcription factors. Since NER activity and gene expression vary by cell-type, it is inherently epigenetically regulated. Furthermore, this epigenetic regulation is disregulated during sporadic breast carcinogenesis. Loss of NER is one basis of genomic instability, a required element in cellular transformation, and one that potentially modulates response to therapy. In this paper, we demonstrate differences in NER capacity in eight adult mouse tissues, and place this result into the context of our previous work on mouse extraembryonic tissues, normal human tissues and sporadic early stage human breast cancer.

INTRODUCTION

Dr. James Cleaver developed the Unscheduled DNA Synthesis Assay (UDS), a functional assay to measure nucleotide excision repair (NER) in attached mammalian cells (1–3). He did this using a UV exposure apparatus that we now refer to as the Cellular Tanning Salon (CTS), shown in Fig. 1, which he designed with Henry Steir at UCSF (4). This instrument delivers a precise dose of UV via a timed, electronic shutter. In addition, Jim developed the scintillation method of measuring UDS that can be used when S phase cells have been nearly eliminated by using hydroxyurea as an inhibitor of replicative DNA synthesis (5). Using these assays, Dr. Cleaver and others were able to establish the link between the

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inherited human disease Xeroderma pigmentosum (XP) and its inherent predisposition to carcinogenesis as due to deficiency of NER (6).

The UDS assay has been applied to toxicity testing on liver cells in vitro (7–9) and in vivo (10). In addition to UDS, several other related assays exist for measuring NER using such techniques as the comet assay (11) and PCR (12). ELISA-based immunoassays are also used for measuring the disappearance of DNA damage (13,14). NER assessment has also been performed with flow cytometry (15), and for transcription coupled repair, with UV-damaged plasmids, either in cell-free extracts (16) or by transient transfection (17,18). Most recently the UDS assay has been adapted to Click-iT chemistry (19), which addresses many of the issues raised by the previous use of BrdU (20,21).

We have previously shown that NER capacity is tissue-specific during embryogenesis (22). We have extended this work (23–25) to the analysis of NER capacity in human tissues. By doing this we have established the “range of normal” for human fibroblasts and epithelial cells from breast and ovarian tissue, which is necessary in a species as outbred as man. It is clear that this range is rather broad and speaks to the possibility that baseline NER capacity in the breast and ovary is modulating risk factor for carcinogenesis.

We have also performed the UDS assay on human breast cells to establish what the non-diseased breast manifests for functional NER capacity, relative to newly diagnosed breast tumors. The baseline repair of non-diseased tissue is critical to the understanding of what changes occur when these cell types undergo oncogenesis. Ironically, transformed cell lines such as Hela cells have been historically used as the standards of comparison in cancer studies, rather than non-diseased cells from the same origin as the tumor. Hela has a very high NER capacity and represents a very advanced tumor type (26).

We believe that the NER pathway is the major pathway of repair for base damage in human DNA. One reason is that NER responds to a gamut of conditions that result in a) inhibition of DNA replication (27), b) inhibition of RNA transcription (28,29), and c) disruption of the DNA helix (30,31). The NER pathway is a large and complicated pathway, consisting of approximately 20 canonical genes necessary for the 5 major steps in the process (damage recognition, incision, excision, replication and ligation), as well as more than 80 more that modify the process, especially the ability to access and monitor non-coding DNA in inactive chromatin (32,33). A detailed and state-of-the-art schematic of this process is provided in the introduction to this special issue (34). The other two base repair pathways remediate replication errors other than DNA damage (mismatch repair) (35) or target specific, common, reiterated damage (alkylation and oxidation) or common replication errors (base excision repair) (36).

It has become possible in recent years for XP patients to live longer, due to UV blocking creams and suits. Longer term, prospective studies of cancer incidence have been performed. NER has long been identified with UV damage and skin cancer (37,38), but internal cancers of the lung, central nervous system, uterus and breast (39,40) have also been identified in XP patients, and hepatocellular carcinomas and lymphoma were characteristic of mouse models of XP (41). This suggests that the NER pathway plays an important role in

remediation of DNA damage in all tissues, not just UV-induced pyrimidine dimers in the skin.

In the field of breast cancer and DNA repair there has been a preponderance of evidence involving homologous recombination repair of double stranded DNA breaks, because of the obvious link to the *BRCA1* gene and the failure of *BRCA1* homozygous mutant cells to perform double strand break repair (42). However, *BRCA1* mutation is only responsible for 5–10% of breast cancers (43). Our work, in contrast, has focused on sporadic (non-germline familial) breast cancer and found that the loss of NER is an intrinsic factor in carcinogenesis of the human breast. This data hearkens back to the fact that NER is modest in the human breast compared with that seen in foreskin fibroblasts (the historical positive standard of comparison used to diagnose NER deficiency syndromes).

The focus of this paper is to place our new data on differential NER capacity in eight adult mouse tissues in the context of similar data on mid-gestation mouse embryos, human adult tissues and the classical DNA repair disease XP. In addition, we will discuss the role of NER deficiency in sporadic (non-germline) breast cancer and show the tumor data in the context of the human classical deficiency syndromes.

MATERIALS AND METHODS

Culture of mouse tissues

Nine tissues, skin, liver, lung, brain, kidney, intestine, spleen, testes and heart were dissected from ICR mice, rinsed in 3X antibiotic/antimycotic in 1X phosphate buffered saline and minced into small pieces with sterile surgical scissors. Minced tissues were placed onto 2-chamber slides with a thin coating of matrigel (matrigel diluted 1:2 in DMEM) (44). Liver cells failed to attach in numbers adequate for analysis. Cultures were maintained for 7 days and then utilized for the UDS assay.

S-phase indices

Primary cultures of mouse tissue, established 7–10 days, were labeled with 10 $\mu\text{Ci ml}$ [^3H]methyl-thymidine ($\sim 80 \text{ Ci mmol}^{-1}$) (PerkinElmer Life Sciences, Boston, MA) for a period of 2 hours at 37°C, followed by a chase of cold thymidine for 2 hours. Slides were then processed for autoradiography, exposed for 10–12 days, developed and analyzed by two independent, blinded scorers who evaluated the tissue samples for the percentage of cells in S phase (characterized by complete coverage of the nucleus with silver grains).

Unscheduled DNA synthesis assay

NER was measured by autoradiography of UDS (3,24). After 7–10 days in culture, without passaging, cultures were irradiated with UV light at 254 nm at a mean fluence of 1.2 Joules/m² for 12 seconds in the absence of culture medium, for a total dose of 14 J/m². Primary cultures had not reached confluence and were still actively growing at the time the UDS assay was performed.

Grain counting

After photographic development of the emulsion on the slides, the nuclei were stained with Giemsa and examined at 1000 $\times$ magnification on a Zeiss Axioskop under emersion oil for grains located immediately over the nuclei of non-S phase cells. For each sample assayed, grains were counted over at least 100 nuclei. Background evaluation and grain processing were performed as previously described (3,24). For the data presented here, 3 control foreskin fibroblast slides were scored, by four independent counters. 200 nuclei were scored per slide, with an average of 25.8 grains/nucleus. For the 8 adult mouse tissues analyzed 47,545 grains were counted by 9 independent counters. An average of 2 slides were scored for each tissue, by four independent counters. An average of 145 nuclei were scored per slide for these samples, ranging from a low of 79 in the skin sample to a high of 200 in the spleen sample. The average grains/nucleus over all mouse tissues was 16.1, with a range from 4.4 in the lung sample to 51.5 in the skin sample (these numbers largely reflect the ratio of irradiated to unirradiated nuclei scored). The distribution of grain counts suggested that each of these cultures consisted of a single major population with regard to DNA repair capacity.

Statistical analysis

In order to prevent transcription errors, raw grain counts from the UDS assay were processed independently in duplicate using the Data Analysis Toolpack of the Excel 2004 spreadsheet program (Microsoft Corp., Redmond, WA). The final count from slides of the same cell type within the same experiment and developed the same day were averaged together and expressed as a percentage of concurrently analyzed foreskin fibroblasts. Comparisons between different cell types were performed using two-tailed t-tests with significance determined at $\alpha < 0.05$. The possible effects of tissue of origin and proliferation in culture (as determined by the S-phase index) were evaluated using multiple linear regression at the same level of significance.

RESULTS AND DISCUSSION

NER capacity in the mouse is tissue-specific

Embryonic mice—Upon analysis (22), several components of the mouse extraembryonic yolk sac had very low global genomic repair capacity as measured by the UDS assay, including visceral and parietal endoderm. Human foreskin fibroblasts exhibited much higher NER capacity than mouse skin fibroblasts, consistent with the theory that UDS capacity corresponds to species longevity (45). In humans, and this is usually extrapolated to other long-lived mammalian species, the genome is constantly surveyed for damage that can be repaired by the NER pathway, including damage in the vast majority of the DNA that is “non-coding”. In rodents (including mice [46], rats [47] and Chinese hamsters [48]), and they are assumed to be representative of mammalian species with shorter life spans, it appears that most NER takes place specifically at transcribed genes, with a lesser ability to repair the “global” genome. However, it is now known that this apparent inherent deficiency in NER in the mouse is secondary to a characteristic difference in the ability of the *DDB2* gene product to bind p53 protein and therefore for the “global” aspect of the NER pathway to be upregulated by DNA damage (49).

Among the mouse tissues, fetal-derived fibroblasts demonstrated higher NER capacity than adult skin fibroblasts, suggesting a loss of repair capacity with age, as has been suggested from human data (50). Most of the extraembryonic tissues studied had very low levels of NER, consistent with their terminal differentiation and short life span. The exception was the extraembryonic mesoderm, which may contribute to the hematopoietic stem cells that migrate to the embryo proper to colonize the embryonic liver. Apparently, these cells are highly vulnerable to genotoxic cell death, since 50% of *XPA* knockout mice die of severe anemia at mid-gestation (51).

Adult mice—We now show that NER capacity is also tissue-specific in the adult mouse. A total of nine tissues were originally selected for analysis: the detoxification organs liver, kidney and spleen; lung and intestine as common sites of cancer; testes, because testicular cancer is commonly treated with *cis*-platinum, which creates intra-strand crosslinks which are remediated by the NER pathway; heart, because it is refractory to carcinogenesis, brain, because there are neurological abnormalities associated with many DNA repair deficiency diseases, including those of the NER pathway; and skin, since human skin is the historical standard of comparison for clinical diagnosis of XP. Liver did not adapt well to our culture system, which uses matrigel as a substrate instead of purified collagen (52). A continuum of repair capacities were found in this limited survey, from absence of repair activity to levels approaching that our traditional control, human foreskin fibroblasts (FF).

Mouse skin was found to have low repair capacity, consistent with our previous study (22). Mouse lung and testes had less than 50% of the activity of human FF and were not significantly different ($P = 0.17$), nor were spleen and kidney ($P = 0.92$), which exhibited slightly greater than 50% of human FF activity. Heart and intestine were not significantly different from one another ($P = 0.54$), and exhibited NER capacities at levels consistent with the FF control. This pattern of NER activity in mouse tissues is not identical to that reported in the rat (53). However, besides the species difference, that study also analyzed transcription-coupled repair, rather than global genomic repair.

The previous results are all broadly relevant to tissue-specific cancer susceptibility. However, we consistently found no evidence of repair activity in mouse brain tissue. Although brain cancer is the second most common childhood cancer in humans, it is relatively rare in adults (54), and these results are in adult mice. There cannot be negligible DNA repair in the human brain because neurological effects are associated with NER deficiency diseases (55). Therefore, if mouse is a model for human at all in this context, then human brain may only exhibit p53-inducible NER, which is then lost with NER gene mutation. The brain is fairly well protected from exogenous damaging agents by the blood-brain barrier (56). The brain should still be susceptible to endogenous damage, however (57), and therefore to the accumulation of mutations we normally associate with carcinogenesis. If brain cells are refractory to transformation, as was initially observed in heart muscle cells (58), then this accumulation may result in functionally defective brain cells, eventually leading to dementia. The phenotypes of the two major NER deficiency diseases, XP and Cockayne syndrome (CS) are usually described as “cancer-prone” and “premature aging”, respectively. Since cancer is a disease of aging, they may just be alternate manifestations of the same underlying defect. The neurological aspects of these

diseases may then also be a symptom of premature aging. The mouse is a problematic model for human aging, however, given that mouse knockouts for CS genes exhibit cancer predisposition (59) and telomerase knockout mice also failed to exhibit a premature aging phenotype for several generations (60).

Although we have never seen any effect of proliferation on NER capacity in our studies, we once again examined this possibility in these adult mouse tissue samples. As seen in Fig. 4, there was absolutely no evidence for any association between NER capacity and proliferation in these samples as indicated by the S-phase index ($P = 0.61$). The hypothesis that proliferation affected DNA repair capacity was developed prior to the discovery of cell-cycle checkpoints, that act to slow or stop DNA synthesis in the face of DNA damage and allow for DNA repair (61,62). In addition, much of the investigation into the molecular mechanisms of mammalian DNA repair have been performed in transformed or tumor-derived cell lines, which have abrogated cell-cycle check points (63). Note, however, that we found no association between NER and proliferation as indicated by S-phase index even in our population of human stage I breast tumors (64).

NER capacity in humans is tissue-specific

Human tissue—We have previously demonstrated differential NER capacity in the human breast, ovary and lymphocytes (Fig. 5, 24, 25). The low level of repair manifested in peripheral blood lymphocytes relative to foreskin fibroblasts may not have a biological impact, because these cells are terminally differentiated and turn over every three months (65). The epithelial cell-types derived from breast and ovarian tissues are not significantly different from one another in NER capacity (26.3 vs. 21.0% of foreskin fibroblast capacity, respectively; $P = 0.27$), however, this level of NER capacity represents a level of repair that may render these tissues vulnerable to helix destabilizing lesions from the environment.

From earlier work on cell lines and peripheral blood lymphocytes, NER capacity has been proposed to be associated with the age and proliferation rate of the cells analyzed (50). We have not found such effects in our studies of human lymphocytes, breast or ovarian epithelial cells (24,25).

Altered NER capacity in human breast tumors

Sporadic human stage I breast tumors—Taking advantage of our ability to grow human tissue in culture, we have also performed UDS on a set of stage I sporadic breast tumors (64). Fig. 6 shows the NER capacity measured in breast tumor and normal primary cultures expressed relative to the mean of these normal breast reduction explant cultures (BRE). The mean NER capacity of the stage I tumor samples was significantly lower than that of BRE, averaging only 44% of normal activity ($P < 0.001$). Multivariate and pairwise analyses revealed no association between tumor NER capacity and patient age at diagnosis, menopausal status, tumor size, nuclear grade, ER and/or PR status, sample cell proliferation (as measured by the S-phase index), recurrence, or disease-free interval. The level of NER exhibited by these stage I sporadic tumors was consistent with those seen in some of the classical NER deficiency syndromes (Fig. 7).

Normal tissues from an individual with BRCA1-deficient hereditary cancer—

Although NER is significantly reduced in stage I sporadic breast tumors, we found normal levels in peripheral blood lymphocytes and uninvolved breast tissue of a *BRCA1* mutation carrier (66). The *BRCA1* gene product is involved in DNA double strand break repair (67), and there is some evidence for a heterozygous genetic instability phenotype at the cellular level (68), so it may be that loss of a single pathway of DNA repair is sufficient to promote carcinogenesis. Alternatively, we have suggested that the intrinsically low levels of NER observed in breast and ovarian epithelium may make them particularly susceptible to carcinogenesis if a second pathway of DNA repair is compromised.

Epigenetic regulation of NER

Analysis of NER gene expression—To further investigate the loss of NER activity observed in stage I breast tumors, we compared the expression of 20 canonical NER genes (69) in 3 tumors versus 3 sample of BRE by gene expression microarray and RNase protection (Fig. 8). All 20 of these core NER genes were expressed at lower levels in the tumor relative to the breast reduction samples by microarray analysis, and 19 of the 20 by RNase protection. Validated monoclonal antibodies were obtained for the products of five NER genes, including four genes shown to be consistently underexpressed in stage I breast tumors by both microarray and RNase protection, as well as for the product of the *ERCC1* gene, which exhibited variable results at the mRNA level. These gene products were then quantified from representative breast reduction and tumor extended explant cultures. Overall, protein levels were highly concordant with the mRNA expression levels as determined by microarray ($P < 0.001$) or RNase protection ($P = 0.007$). Significantly lower levels of XPA and CSB proteins (both $P < 0.001$) were observed in the tumor, as well as DDB2 (XPE) protein ($P = 0.004$). The lower protein amount for DDB1 in the tumor failed to reach statistical significance ($P = 0.45$), as did the slight increase in ERCC1 protein ($P = 0.79$). *ERCC1* was the only core NER gene to show somewhat inconsistent overexpression at both the mRNA and protein level. This apparent lack of consistent down-regulation of ERCC1 may be specific to this gene, as down-regulation of its partner protein XPF would be sufficient to lower its activity. With the exception of a single, severely affected individual (70), the null phenotype for ERCC1 is not found in human XP disease states, so the expression of this gene below a certain level may not be generally viable (71).

In summary, several independent lines of evidence point to the fact that the NER DNA repair pathway and its major genes are epigenetically regulated:

1. Functional NER capacity is tissue-specifically regulated during mammalian embryogenesis and in the adult mouse and human.
2. Functional NER capacity is deficient in sporadic (unexplained) stage I breast cancer, i.e. is disregulated upon cellular transformation
3. The expression of most or all of the canonical NER genes are coordinately down-regulated in stage I tumors relative to breast reduction mammoplasty cultures. This steady state expression is durable at the level of translation.

These observations are in addition to previous studies, including our own, showing that NER activity and gene expression can be altered by DNA demethylating agents (72,73).

Our discussion of the implications of a lack of NER expression in the mouse brain should not be considered definitive. Cleaver et al. (74) have just published a study suggesting that the neurological symptoms of NER deficiency are solely based on damage to the mitochondria. This possibility is consistent with the phenotypes of mitochondrial diseases affecting oxidative phosphorylation and inducing somatic mitochondrial DNA mutations (75).

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Fig. 1. UV exposure apparatus or “Cellular Tanning Salon” developed by James Cleaver and Henry Steir at UCSF. This instrument delivers a precisely timed dose of UVC via an electronic shutter, and delivers it evenly because of the use of a turntable (3,4).

Fig. 2.

Summary of the mean UDS levels of a subset of embryonic and extraembryonic murine lineages expressed as a percentage of human foreskin fibroblasts (FF). Error bars represent standard error. Two-tailed pairwise Student's *t* tests between all six murine cell-types (adult skin, fetal fibroblasts, extraembryonic mesoderm, visceral endoderm, trophoblast and parietal endoderm) indicated that there were statistically significant differences (at $P < 0.0001$) in all pairwise permutations except between visceral endoderm and adult skin ($P = 0.08$), visceral endoderm and trophoblast ($P = 0.27$) and parietal endoderm and trophoblast ($P = 0.33$). From Latimer et al. (22).

Fig. 3. NER capacity of cultured adult mouse tissues, as determined with the functional UDS assay. As explained in the text, lung and testes, spleen and kidney, and intestine and heart all represent pairs of tissues with equivalent NER capacities. Skin is significantly lower in repair than all other tissues except lung ($P = 0.14$), Lung/testes is significantly lower in NER capacity than either spleen/kidney or heart/intestine (both $P < 0.001$). Spleen/kidney is also significantly lower than heart/intestine ($P = 0.019$).

Fig. 4.

Lack of proliferation-dependence on NER capacity in primary cultures of various adult mouse tissues. Multiple linear regression demonstrated no significant effects of tissue of origin or proliferation in culture (as determined by the S-phase index) on NER capacity ($P < 0.05$).

Fig. 5. NER capacity of adult human cell-types, as determined with the functional UDS assay., After pooling of the non-significantly different data from the breast- and ovarian-derived epithelium, these tissues are both significantly higher than the capacity of peripheral blood lymphocytes, and lower than foreskin fibroblasts ($P < 0.001$ for both). Foreskin fibroblasts and peripheral blood lymphocytes are also significantly different in NER capacity in this analysis ($P < 0.001$). From Latimer et al. (25).

Fig. 6. NER capacity of breast reduction epithelium (BRE) and stage I breast tumors expressed relative to average breast reduction explant culture. Comparison of NER capacities of primary explant cultures established from breast reduction mammoplasty tissue from nondiseased controls (N = 23) and stage I ductal carcinoma (N = 19). The asterisk (*) indicates that tumor repair capacity is significantly lower than control (breast reduction epithelial explant cultures) at $P < 0.05$. From Latimer et al. (64).

Fig. 7. Observed NER capacities for breast reduction mammoplasty (normal breast tissue (in red), breast tumors stage I (in yellow)). The range of NER capacities for various XP deficiency disorders are shown in green. It is clear that the tumors are in the most deficient range of NER capacity seen in humans and that the lower end of normal breast tissue NER activity also appears to be relatively deficient. All samples are expressed in terms of FF repair capacity.

Fig. 8. Molecular analysis of NER in nondiseased breast reduction and stage I breast tumor explant cultures. (A) Microarray analysis of NER using the Affymetrix U133 2.0 Plus chip processed using the Probe Logarithmic Intensity Error algorithm. Summary of results for 20 core NER genes shows reduced mRNA expression in tumor (solid bars, N = 3) versus age-matched normal breast explants (open bars, N = 3). The observed reduced levels of expression observed in stage I tumors were individually significant for nine genes: *TFIIHp44* (P = 0.024), *RPAP32* (P = 0.031), *XPD* (P = 0.013), *CSA* (P = 0.046), *CDK7* (P =

0.026), *DDB2* ($P = 0.035$), *DDB1* ($P = 0.0002$), *hHRAD23B* ($P = 0.032$), and *RPAp70* ($P = 0.036$); and came close to significance for four others: *TFIIHp34* ($P = 0.054$), *XPC* ($P = 0.093$), *XPF* ($P = 0.060$), and *XPG* ($P = 0.070$). (B) Quantitative RNase protection analysis of 20 canonical NER genes in nondiseased breast reduction epithelial and stage I breast tumor explant cultures. Gene expression for all genes except *ERCC1* is significantly lower ($P < 0.05$) in breast tumor (solid bars) than in normal breast explants (open bars). Data given and variability are derived from nine to 14 individual analyses for each gene. (C) Western analysis analyses of five NER proteins in nondiseased breast reduction epithelial and stage I breast tumor explant cultures. Protein levels were reduced for the XPA, CSB, DDB1, and DDB2 gene products in breast tumor (solid bars) relative to normal breast explants (open bars). These reductions were significant for the XPA, CSB, and DDB2 proteins as detailed in the text. Data given and variability are derived from three replicate analyses for each protein. From Latimer et al. (64). For all panels, * indicates tumor expression significantly lower than control (breast reduction epithelial explant cultures) at $P < 0.05$, whereas (*) in panel A indicates $0.05 < P < 0.1$.