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Cell-type-specific level of DNA nucleotide excision repair in primary human mammary and ovarian epithelial cell cultures

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Abstract

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DNA repair, a fundamental function of cellular metabolism, has long been presumed to be constitutive and equivalent in all cells. However, we have previously shown that normal levels of nucleotide excision repair (NER) can vary by 20-fold in a tissue-specific pattern. We have now successfully established primary cultures of normal ovarian tissue from seven women by using a novel culture system originally developed for breast epithelial cells. Epithelial cells in these cultures aggregated to form three-dimensional structures called “attached ovarian epispheres”. The availability of these actively proliferating cell cultures allowed us to measure NER functionally and quantitatively by the unscheduled DNA synthesis (UDS) assay, a clinical test used to diagnose constitutive deficiencies in NER capacity. We determined that ovarian epithelial cells manifested an intermediate level of NER capacity in humans, viz., only 25% of that of foreskin fibroblasts, but still 2.5-fold higher than that of peripheral blood lymphocytes. This level of DNA repair capacity was indistinguishable from that of normal breast epithelial cells, suggesting that it might be characteristic of the epithelial cell type. Similar levels of NER activity were observed in cultures established from a disease-free known carrier of a *BRCA1* truncation mutation, consistent with previous normal results shown in breast epithelium and blood lymphocytes. These results establish that at least three “normal” levels of such DNA repair occur in human tissues, and that NER capacity is epigenetically regulated during cell differentiation and development.

Keywords

Primary ovarian epithelial culture; Nucleotide excision repair; Unscheduled DNA synthesis; Xeroderma pigmentosum; *BRCA1*; Human

Introduction

Living organisms are subjected to a constant barrage of DNA damage caused by exposure to a variety of exogenous chemical and physical agents and by endogenous metabolic processes. Unrepaired DNA damage leads to the accumulation of somatic mutation and the eventual development of neoplastic transformation. One of the pathways to repair DNA damage before it can result in mutation is long-patch or “nucleotide excision” repair (NER). NER is a complex pathway involving five discrete steps requiring the products of over 30 genes (van Hoffen et al. 2003). It is primarily identified with the repair of cyclobutane pyrimidine dimers, 6-4 photoproducts, and DNA cross-links caused by UV light; however, the NER pathway also provides redundant support for other repair mechanisms, since it is activated by any distortion in the DNA helix (Hanawalt et al. 2003).

Constitutive deficiency in NER capacity results in the cancer-prone disease xeroderma pigmentosum (XP; Cleaver 2005). The clinical diagnosis of XP is based on direct and quantitative measurement of NER of the overall genome by using the unscheduled DNA synthesis (UDS) assay in either skin fibroblasts or peripheral blood lymphocytes. The UDS assay quantifies labeled base incorporation into the DNA after in vitro exposure to a specific dose of UV light. The UDS assay is a cell-autonomous in situ functional assay in that it allows for the analysis of the complex process of NER as a whole, inclusive of both transcription-coupled repair and bulk global genomic repair. However, it requires living metabolizing cells for label incorporation following exposure and DNA damage.

Studies involving functional assays of DNA repair capacity (and functional assays in general) have been hampered by a technical inability to perform primary explant culture on many cell types. In particular, lineages that involve epithelial cells have proven to be extremely difficult to culture, perhaps because these cells normally rest on a complex, biologically reactive, basement membrane produced in vivo (Engel and Young 1978). We have developed a novel system for establishing primary cultures of breast epithelial cells (Latimer 2000) and, as we now report, ovarian epithelial cells. Since most tumors arise from epithelial cells, the evaluation of their baseline mutator gene functions, including intrinsic DNA repair capacity, is extremely important.

We have previously shown that NER capacity in the mouse is lineage-specific during embryogenesis (Latimer et al. 1996), and that normal human breast epithelial cells manifest an intermediate level of NER capacity between those exhibited by skin fibroblasts and peripheral blood lymphocytes (Latimer et al. 2003). The hereditary breast and ovarian cancer gene *BRCA1* is mostly associated with DNA double-strand break repair (Rosen et al. 2003), but it has also been shown to modulate NER activity (Hartman and Ford 2002; Takimoto et al. 2002). In this study, we apply the functional UDS assay to assess the intrinsic NER capacity of non-diseased ovarian epithelial cells established within a novel epithelial cell culture system. These cells exhibit an NER capacity indistinguishable from those of breast epithelial cells, suggesting that an intermediate level of NER capacity may be intrinsic to epithelial cell types, possibly contributing to their susceptibility to transformation and tumorigenesis.

Materials and methods

Tissue procurement and establishment of cultures

Ovaries were obtained from patients at Magee-Womens Hospital and Evanston Northwestern Hospital under IRB MWH94-108. These patients underwent hysterectomy for either benign gynecological conditions or for cancer unrelated to the ovary. All ovaries were determined pathologically to be normal.

Ovarian epithelium was harvested by gentle physical scraping of the single cell layer of the epithelium with a scalpel. Cells were then rinsed off the stromal center of the ovary and centrifuged into a pellet. This pellet was disaggregated by gentle trituration with a small volume of medium and plated directly onto single chamber slides (Lab-Tek, Thermo Fisher Scientific, Waltham, Mass.) pre-coated with diluted (1:1 with DMEM) Matrigel (BD Biosciences, San Jose, Calif.), containing MWRI α medium (Latimer 2000; Latimer et al. 2003). All ovarian samples were processed and placed into primary culture within 5 h of surgery.

Primary ovarian cultures were grown for 7–10 days, digitally imaged, and then analyzed for NER capacity by using the UDS assay. UDS experiments were performed when the epithelial cells were present as clusters of epithelial cells numbering from 40–150 cells, similar to objects that we refer to in breast epithelial cell culture as attached “epispheres”.

Newborn foreskins, peripheral blood lymphocytes, and breast-reduction mammoplasty tissues were obtained and processed for culture and UDS analysis as previously described (Latimer et al. 2003). Subjects used for generation of foreskin fibroblast cultures were all younger than 3 days old. Foreskin fibroblasts were grown in MEM containing 10% fetal calf serum on uncoated chamber slides and analyzed between passages 7 and 10. Donors of peripheral blood lymphocytes were recruited from laboratory and hospital workers and ranged from 20 to 50 years of age ($n=33$). These cells were isolated by Ficoll gradient, cultured in RPMI medium with 15% fetal calf serum, and analyzed after 5–7 days in culture without passaging (Forlenza et al. 2000; Latimer et al. 2003). Breast-reduction mammoplasty tissues were obtained from patients at Magee-Womens Hospital under Magee-Womens Hospital/University of Pittsburgh IRB no. MWH-94–108. These women ranged in age from 19 to 64 years ($n=23$). Histological evaluation of breast tissue adjacent to that placed into culture demonstrated that none of the samples selected for this study contained changes that were outside of the range of normal (Latimer et al. 2003, 2005).

S-phase indices

Primary cultures of ovarian tissue, established for 7–10 days, were labeled with 10 $\mu\text{Ci ml}^{-1}$ [^3H]methylthymidine ($\sim 80 \text{ Ci mmol}^{-1}$; PerkinElmer Life Sciences, Boston, Mass.) for a period of 2 h at 37°C, followed by a chase of cold thymidine for 2 h. 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).

UDS assay

NER was measured by autoradiography of UDS and expressed as a fraction of the concurrently analyzed foreskin fibroblast control (Latimer et al. 2003; Kelly and Latimer 2005). After 7–10 days in culture, without passaging, cultures were irradiated with UV light at 254 nm at a mean fluence of 1.2 J/m² for 12 s 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 that the UDS assay was performed. Slides were then incubated for 2 h in medium containing 10 $\mu\text{Ci ml}^{-1}$ [^3H]methylthymidine at 37°C, followed by 2 h in unlabeled medium, fixed, dried, dipped in photographic emulsion, and exposed for 10–12 days in complete darkness at 4°C. Positive control (foreskin fibroblast) “tester” slides were developed at 10 days and used to determine the optimum exposure period necessary to maintain a relatively consistent number of silver grains/nucleus across experiments.

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 from clearly identified epithelial cells composing the flattened upper surface of ovarian epispheres. Background evaluation and grain processing were performed as previously described (Latimer et al. 2003; Kelly and Latimer 2005). For the data presented here derived from five independent experiments, an

average of five foreskin fibroblast slides was scored per experiment, by three to four independent counters. Per slide, 150 nuclei were scored, with an average of 33.4 grains/nucleus. An average of four slides was scored for the primary ovarian cultures, by two to three independent counters. Again, 150 nuclei were scored per slide for these samples, but the average grains/nucleus was only 9.4. 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, once with StatView (version 5.0.1, SAS Institute, Cary, N.C.) and once with the Data Analysis Toolpack of the Excel 2001 spreadsheet program (Microsoft, Redmond, Wash.). The final count from slides of the same cell type within the same experiment and developed the same day was averaged and expressed as a percentage of concurrently analyzed foreskin fibroblasts. Comparisons between different cell types were performed by using two-tailed *t*-tests with significance determined at $\alpha < 0.05$. The possible effects of tissue of origin, cell type, donor age, and cell proliferation in culture (as determined by the S-phase index) were evaluated by multiple linear regression at the same level of significance.

Results

Primary culture of ovarian epithelium

These experiments were designed to analyze one specific type of DNA repair, NER, in ovarian epithelial cells and to compare their repair capacity with those of skin fibroblasts, peripheral blood lymphocytes, and non-diseased human breast epithelium. NER was measured by using the clinically applied functional UDS assay, a technique requiring attached, live, and actively proliferating cells for its application (Kleijer et al. 2007).

Primary ovarian tissue was derived via hysterectomy from six patients: four with benign gynecological problems (three with myometrial fibroids, one with endometriosis) and two with cervical cancer not involving their ovaries (one squamous cell carcinoma of the cervix, one mucinous adenocarcinoma arising from a polyp). These patients ranged in age from 43 to 52 (average of 47.3) years.

A tissue-processing and culture protocol was developed for ovarian samples based on our successful tissue engineering of breast epithelium (Latimer et al. 2003). The salient features of this protocol involved physical scraping of the ovarian epithelium, physical disaggregation of the resultant tissue with no additional attempt to purify epithelial cells, and growth on a commercially available substrate in medium with high serum content (Latimer 2000). Freshly scraped ovarian epithelial cells and a small number of fibroblastic cells adhered to the substrate within 24 h. Ovarian epithelium manifested in this culture system initially as foci of rounded cells in “cobblestone” monolayers, which quickly clustered to form three-dimensional structures that we call “attached ovarian epispheres” (two examples are presented in Fig. 1) after the similar structures formed in primary cultures of human

breast reductions (Latimer et al. 2003). Primitive epispheres were evident as early as 48 h after plating but began to dominate the culture after 7–10 days.

Since our culture system was designed and optimized for growth of mammary epithelial cells, a comparison of its support of ovarian epithelial cultures is of interest. One measure of cell growth and viability is the S-phase index or the percentage of cells incorporating radiolabeled thymidine into DNA during replication (“scheduled” as opposed to “unscheduled” or repair synthesis) over a specific incubation time (in our case, 2 h). Our previous experience with breast epithelial cells demonstrated a wide range of S-phase indices, from 0.2% to 46%, with a mean $\pm$ SE of $18.9\pm 2.5\%$, $n=23$ (Latimer et al. 2003, 2005). The six normal ovarian samples cultured for these studies had a similar range of proliferation rates, with S-phase indices from 4.4% to 29%, and an average value not significantly different from those of the breast epithelial cultures, $16.0\pm 2.9\%$ ($P=0.58$).

NER capacity of primary ovarian cultures

UDS analysis of six non-diseased primary ovarian epithelial cultures yielded an NER capacity of $20.2\pm 2.4\%$ of the concurrently analyzed foreskin fibroblast controls, with a coefficient of variation of 11.9% (Fig. 2). This value was significantly higher than that of peripheral blood lymphocytes ($P=0.00006$) and significantly lower than the NER capacity of foreskin fibroblasts themselves ($P=0.00002$; although this was defined as 100% in each individual experiment, variability was calculated through comparison with a second control, viz., the breast cancer-derived cell line MDA-MB231). The repair capacity of the six ovarian primary cultures was not, however, significantly different from the values previously obtained from breast epithelial cultures ($25.8\pm 2.3\%$ of foreskin fibroblasts, $P=0.25$). Indeed, a regression between NER capacity and tissue of origin was highly significant in any case ($P<0.00001$), but the correlation coefficient rose slightly from 0.71 to 0.77 if the breast and ovarian values were combined into a single cell-type category, suggesting that they were equivalent.

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. We have not found such effects in our breast epithelial cells (Latimer et al. 2003, 2005), and we now report the same lack of effect of age ($P=0.14$) and S-phase index ($P=0.82$) on NER levels in ovarian epithelial cultures (although, admittedly, we have a limited age range; Fig. 3). In keeping with the previous paragraph, if the data from breast and ovarian epithelial cultures are combined, these trends persist (age, $P=0.52$; S-phase index, $P=0.41$).

Growth and NER capacity of ovarian epithelial cells from a *BRCA1* gene mutation carrier

We have previously shown that the peripheral blood lymphocytes and non-diseased breast tissue from a breast cancer patient who was a *BRCA1* mutation carrier both showed tissue-appropriate levels of NER (Latimer et al. 2005). A 37-year-old disease-free female relative of this patient was found also to carry the same Q1200X truncation mutation in one of her *BRCA1* alleles and opted for preventive procedures, including prophylactic oophorectomy. Since *BRCA1* mutations predispose to both breast and ovarian cancer (Narod 1994), we

obtained and cultured samples of tissue from both ovaries and analyzed them for NER capacity with the functional UDS assay.

The S-phase indices measured in the primary cultures from these ovaries were 27% from the left ovary and 21% from the right. Both values were within our range of normal samples, but both were somewhat higher than average. These cancer-predisposed ovarian samples grew well in our culture system, showing no evidence that haploinsufficiency for *BRCA1* affected the viability and growth of normal ovarian epithelial tissue, consistent with our experience with breast epithelium (Latimer et al. 2005).

The NER capacities of these samples also appeared to be tissue-appropriate and well within the range of normal, with the left ovarian sample demonstrating a repair capacity of 15.9% of foreskin fibroblasts and the right ovarian sample exhibiting a repair capacity of 21.3% of foreskin fibroblasts (Fig. 4). Although close, these two results were not as similar to one another as the repair capacities measured in samples from the ipsilateral and contralateral breasts of the woman's affected relative (Fig. 4; Latimer et al. 2005).

In keeping with their apparently normal levels of DNA repair, inclusion of these *BRCA1* haploinsufficient ovarian, breast, and peripheral blood samples did not significantly affect any of the tissue-specific, age and proliferation rate analyses reported in the previous section.

Discussion

Although the successful primary culture of ovarian epithelial cells has been previously reported (for example, Auersperg et al. 1997), most of these cultures converted to a more mesenchymal/fibroblastic morphology after only a few passages on tissue culture plastic. Thus, primary ovarian epithelial cultures have not been extensively used to investigate, for example, the development of genomic instability that presages neoplastic transformation. Our finding that ovarian epithelial cells manifest a tissue-specific intermediate level of NER may have important implications with regard to susceptibility to induced mutation and carcinogenesis. The concept of tissue-specific regulation of DNA repair has taken some time to be accepted, because it implies that certain tissues are inherently at greater risk from certain genotoxic insults. For example, if skin fibroblasts manifest full NER capacity, consistent with the primary role of NER in repairing UV-induced DNA damage, then breast and ovarian epithelium, at 20%–25% of that activity are actually in the range of patients with XP, a disease with an inherited predisposition to cancer. Indeed, as improvements in clinical management increase the lifespan of XP patients, it is becoming clear that they are also at risk for epithelial neoplasia (Kraemer et al. 1984; Milo et al. 1994). Martin et al. (1996) have demonstrated that kidney epithelial cells have a significantly higher baseline mutation frequency at the *HPRT* locus than other tissues, an observation that would be consistent with reduced DNA repair. Thus, constitutively low NER may be a general characteristic of epithelial cell types.

Cell-type specificity for NER capacity implies that DNA repair can be regulated epigenetically, since this is the major mechanism of eukaryotic differentiation (Tada and

Tada 2001). This epigenetic regulation paradigm in non-diseased tissue is distinct from those shown in most studies of NER that focus on hereditary and somatic deleterious mutations. Moreover, the observation that both breast and ovarian epithelium is naturally and contextually deficient in NER provides a possible explanation for the tissue specificity of tumors arising because of *BRCA1/2* mutations: loss of double-strand break repair would be more deleterious in cell types that manifest reduced levels of other types of DNA repair (Latimer et al. 2003, 2005). An intermediate level of NER in epithelial cells allows for both increases and decreases in this type of DNA repair through purely epigenetic mechanisms, such as the global changes in DNA methylation that often occur during carcinogenesis (Ting et al. 2006).

Several studies have now associated constitutively low levels of NER, presumably genetically determined, with breast cancer incidence (Kovacs et al. 1986; Kovacs and Almendral 1987; Xiong et al. 2001; Kennedy et al. 2005). In ovarian cancer, interest in the NER pathway has focused on its primary role in the remediation of intra-strand DNA crosslinks (ICL), a type of DNA damage caused by *cis*-platinum, the major chemotherapeutic agent used for this disease (Reed 1998). Although these studies have been based largely on measuring the expression of only one or a few of the many NER genes, an association has generally been seen between gene upregulation and drug resistance (Ferry et al. 2000; Li et al. 2000). Moreover, this clinically significant alteration in NER gene expression appears to occur epigenetically (States and Reed 1996; Yu et al. 2000). Thus, our data suggest a facile epigenetic mechanism for both loss of NER capacity in epithelial cells, which could contribute to genomic instability during carcinogenesis, and gain of repair capacity, such as occurs in drug-resistant advanced-stage ovarian tumors.

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Fig. 1.

Primary culture of ovarian epithelial cell samples. After 7–10 days in culture, most epithelial cells aggregate into large three-dimensional structures called attached ovarian epispheres. **a** A distinct single symmetrical unit. **b** An example of a more complex compound structure with two epispheres joined by an epithelial bridge. Silver grains over the nuclei in these structures were quantified to provide the data from the UDS assay reported in Fig. 2. Optical DIC (differential interference contrast) images from a Zeiss Axiovert 100 microscope captured with a high-resolution Hamamatsu C4742–95 cooled charge-coupled device digital camera

Fig. 2. NER capacity of the different cell types, as determined with the functional UDS assay. NER capacity of foreskin fibroblasts (FF; arbitrarily set at 100%), primary cultures of breast epithelial cells ($24.7 \pm 2.2\%$), primary cultures of ovarian epithelial cells ($20.9 \pm 2.5\%$), and peripheral blood lymphocytes ($8.9 \pm 1.2\%$) from normal healthy women

Fig. 3. Lack of (a) age-dependence and (b) proliferation-dependence on NER capacity of primary breast (open circles) and ovarian (open squares) epithelial cell cultures. Filled symbols indicate results from heterozygotes for a Q1200X mutation at the *BRCA1* locus represented by the contralateral breast tissue of a breast cancer patient (Latimer et al. 2005) and by the right ovary of a disease-free relative of this patient. Solid lines represent the linear regression of the ovarian data, long-dashed lines represent the linear regressions of the breast data, and

short-dashed lines represent the pooled epithelial cell data. None of these regressions is statistically significant. *FF* foreskin fibroblasts

Fig. 4. NER capacity of primary breast and ovarian epithelial cell cultures from individual normal women (*open symbols*) and from women heterozygotes for mutation at the *BRCA1* locus. *Filled black circles* indicate results from the contralateral (upper) and ipsilateral (lower) breast tissue of a breast cancer patient who carried a Q1200X *BRCA1* mutation. *Filled gray squares* represent the right (upper) and left (lower) ovaries of a disease-free relative of the above patient who opted to have a bilateral prophylactic oophorectomy. *Open squares* represent the NER capacity of six primary cultures (from six different individuals) derived from non-diseased ovaries (*FF* foreskin fibroblasts)