Oxidative Damage to DNA of Ovarian Surface Epithelial Cells Affected by Ovulation: Carcinogenic Implication and Chemoprevention

WILLIAM J. MURDOCH*,[1] AND JAMES F. MARTINCHICK†

*Department of Animal Science and Reproductive Biology Program, University of Wyoming, Laramie, Wyoming 82071; and †Department of Surgical Pathology, Ivinson Memorial Hospital, Laramie, Wyoming 82072

The majority of cancers of the ovary are thought to originate from a surface epithelial cell perturbed by ovulation. Outgrowth of a follicle destined to ovulate brings it into apposition with the ovarian epithelium. Ovarian surface cells are consequently exposed, within a limited diffusion radius, to inflammatory agents and reactive oxidants generated during periovulatory processes. Cells that overlie the formative site of follicular rupture suffer irreparable damages and undergo apoptosis. Potentially mutagenic 8-oxoguanine modifications were detected in (surviving) cells circumjacent to postovulatory ovine and human follicles. It is conceivable that clonal expansion of a cell with unrepaired DNA, but not committed to death, could be an initiating factor in the etiology of malignancy, insofar as proliferative ovulatory wound-repair responses may propagate mutations. Since the prognosis for ovarian cancer patients with invasive disease is so poor, and early detection has proven elusive, it is imperative that prospective methods of chemoprevention be explored. Ovulation-induced oxidative base damages to the ovarian epithelium of ewes were prevented by vitamin E. Oxoguanine adducts persisted and CA-125 (a phenotype of metaplastic transformation) was expressed in cultures of cells that were distressed by ovulation in which p53 synthesis was inhibited. Vitamin E negated this reaction. Ovarian cyclicity and fertility were not altered in vitamin-treated ewes. A prophylactic benefit of a supplemental antioxidant is suggested in "ovulating" individuals designated at risk (e.g., due to a tumor suppressor malfunction) for the development of ovarian cancer. Exp Biol Med 229:546–552, 2004

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[1] To whom correspondence should be addressed at Department of Animal Science, 1000 East University Avenue, University of Wyoming, Laramie, WY 82071. E-mail: wmurdoch@uwyo.edu.

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Mammalian ovaries are encased by a simple cuboidal to a low pseudostratified columnar layer of epithelial cells. Surface epithelial cells are supported along the ovarian cortical interstitium (tunica albuginea) by a basal lamina and are held together laterally by desmosomes and gap/tight junctional complexes (1–4). A follicle selected to ovulate will emerge to the ovarian surface. Ovarian epithelial cells within the immediate vicinity of the ovulatory stigma are subjected to inflammatory mediators and reactive oxygen species produced during the mechanics of follicular rupture (5–9), become apoptotic, and are sloughed (10). Bystander epithelial cells, which surround the site of ovulation, are exposed to sublethal concentrations of oxidants (11); these "stem" cells eventually (upon luteal regression/progesterone withdrawal and transition into the next proliferative/follicular phase) mend the ovarian "wound" (12, 13). There is a general consensus that oxidative damage products in DNA are a major contributor to the risk of cancer development (14).

Most ovarian cancers (>90%) are derived from the ovarian surface epithelium (15–18). Common (i.e., surface) epithelial ovarian cancer has been related to (the trauma of) ovulation. Indeed, circumstances that prevent ovulation (e.g., oral contraceptives and pregnancy) protect against ovarian cancer (19–22). Fortunately, oxidative disturbances to DNA perpetrated by ovulation are generally reconciled during the ensuing luteal phase as a result of p53-dependent cell-cycle arrest and base-excision repair mechanisms (11)—it is a unifocal "escape" that could be problematic (23). Hence, predisposition to ovarian cancer likely involves a defective tumor suppressor/DNA repair pathway. More than one half of human ovarian adenocarcinomas have discernible mutations in p53 (24).

Normal ovarian surface epithelial cells are of an uncommitted phenotype. Cell-surface expression of the glycoprotein cancer antigen CA-125 (25) occurs with differentiation into a Mullerian-type epithelium. Precursor lesions of malignancy evidently emanate from epithelial

cells that have undergone Mullerian metaplasia (26). CA-125 was expressed in cultures of p53-deficient sheep ovarian epithelial cells containing damaged DNA (27).

Epithelial ovarian cancer is a deadly, insidious disease because it typically remains asymptomatic until it has advanced into the abdominal cavity (28–30); therefore, chemoprevention is a high priority. We hypothesized that vitamin E could be utilized to downregulate the genotoxic side effects of ovulation without compromising fertility. As far as is known, vitamin E is the most effective (by acting as a hydrogen donor at its 6-OH group), chain-breaking antioxidant in cellular membranes, and thereby contributes to membrane phospholipid stability and safeguards intracellular molecules against damage imposed by free radicals; it is essentially nontoxic (31–33). There is epidemiological evidence suggesting an inverse relationship between consumption of vitamin E and risk of ovarian carcinoma (34, 35). Similar reports have advocated protective effects of vitamin E against cancers of the lung, stomach, colorectum, cervix, prostate gland, and breast (36–38).

Research related to ovulation and fidelity of ovarian epithelial DNA has to date been conducted using an ovine model (11, 27). An initial comparative study was carried out to characterize oxidative DNA base damages in ovarian surface cells relative to sites of ovulation in sheep and humans. The 8-oxoguanine adduct, an effector of transversion mutagenesis, was used as a marker of base oxidations (39). A prospective antioxidant action of d-α-tocopherol (natural-source vitamin E) was then tested in sheep, and a follow-up experiment was performed to assess its effect on 8-oxoguanine accumulation and CA-125 expression in cells incubated with an antisense p53 oligonucleotide. Preovulatory gonadotropin secretion, ovulation, luteal function, and lambing rates were monitored in vitamin-treated ewes.

Materials and Methods

Experiments were performed with the approval of the University of Wyoming Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Spatial Alterations in Immunoreactive 8-Oxoguanine in Surface Epithelial Cells Isolated from Postovulatory Sheep and Human Ovaries. Mature Western-range ewes were observed twice daily for estrous behavior in the presence of vasectomized rams. Day 0 was considered the first day of estrus (the preovulatory surge of gonadotropins occurs on Day 0; ovulation occurs approximately 24 hrs after the initiation of the surge; Ref. 40). Ovaries containing an ovulated follicle ($n = 4$) were excised at sacrifice (intravenous Beuthanasia-D; Schering-Plough Animal Health, Kenilworth, NJ) at 60 hrs after detection of estrus (Day 2). Early luteal phase ovarian specimens were obtained from women ($n = 3$) that had undergone prophylactic oophorectomy.

Ovaries were fixed by immersion in Histochoice (Amersco, Solon, OH). Ovarian surface epithelial cells were removed (>95% purity) from the perimeters of postovulatory follicles (~3 mm outward) and an extrinsic (i.e., unaffected) area (>10 mm from an ovulation papilla) using a modified polytetrafluoroethylene scraper as described previously (41). Samples were transferred onto microscope slides treated with subbing solution (0.025% chromium potassium sulfate, 0.25% gelatin); air-dried; washed in phosphate-buffered saline (PBS); permeabilized in ice-cold methanol (70% for 3 mins, 90% for 3 mins, 99% for 30 mins); rehydrated to PBS; and analyzed by indirect immunofluorescence microscopy. Slides were incubated for 30 mins with 10% normal goat serum and for 1 hr with an 8-oxoguanine monoclonal antibody (1 μg/ml IF7/4355-MC-100; Trevigen, Gaithersburg, MD); washed in two changes of PBS; incubated for 30 mins with secondary goat antimouse immunoglobulin G-fluorescein isothiocyanate (1:40 F 0257); and washed in two changes of PBS. Images of individual cells were captured (×400; subsampling = 20) by computer-interfaced digital photography (1.2 million pixel resolution; Pixera, Los Gatos, CA) and assessed for luminance intensities (continuous inverted gray scale = 0 [black] to 255 [white]; Optimas Software, Bothell, WA). Negative controls were conducted without primary antibody and with primary antibody preabsorbed with an 8-oxoguanine nucleotide (Trevigen).

Effects of Vitamin E on Ovulation/Luteinization and Oxidant Insults to DNA of the Ovarian Epithelium. Ten ewes per group were treated by intramuscular injection at the onset of estrus with vitamin E (2100 IU; Schering-Plough) or vehicle (7 ml Tween-80 polyoxyethylene sorbitan monooleate + 20% ethanol). The dose of vitamin E was 1.5–2 times above that recommended for treatment of deficient ewes or finishing lambs (31). Serum vitamin E concentrations peak at about 10 hrs and remain elevated for 72 hrs following injection (42, 43). Blood samples for serum luteinizing hormone (LH) radioimmunoassay (44) were obtained by jugular venipuncture at 0, 1, 2, 4, 8, and 16 hrs post-treatments.

Ovarian surface epithelial cells were harvested on Day 2 and analyzed for 8-oxoguanine. Tissue blocks containing a postovulatory follicle were fixed in Histochoice, washed in PBS, dehydrated, cleared, infiltrated with paraffin wax, serially cross-sectioned at 6-μm thickness, floated onto microscope slides, air-dried, deparaffinized in xylene, rehydrated, stained in hematoxylin and eosin, and examined by light microscopy.

***In Vitro* Responses of p53-Attenuated Cells Following *In Vivo* Exposure to Vitamin E.** Ten ewes were treated at detection of estrus with vitamin E or injection vehicle ($n = 5$), and ovarian surface epithelial cells were recovered on Day 2 from circumferences of ovulated follicles and extrinsic zones. Cells were divided equally into aliquots of 0.1 ml RPMI-1640 medium and incubated in 96-well plates for 2 or 6 days (37°C) with 1 μM sodium salt

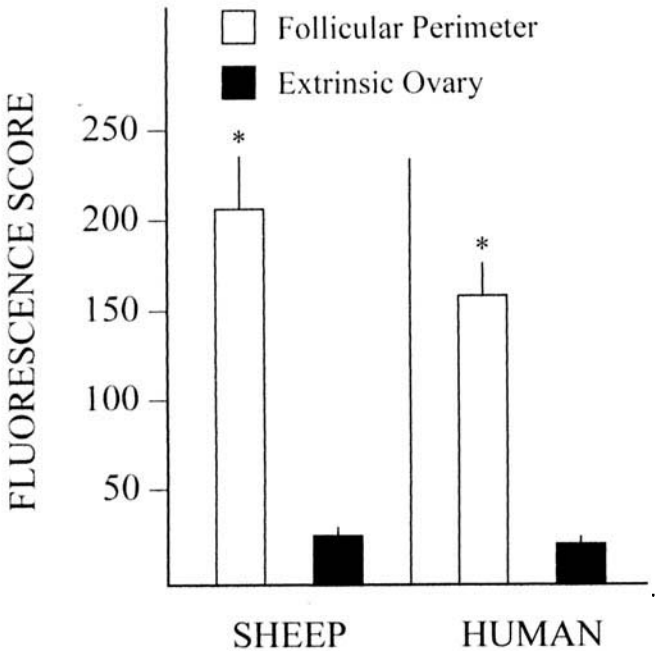

Figure 1. Accretion of 8-oxoguanine in surface epithelial cells isolated from postovulatory ovaries. Means ± SE are plotted. Asterisk (*), $P < 0.01$. Fluorescence intensity values for negative (background) control cells were <22.

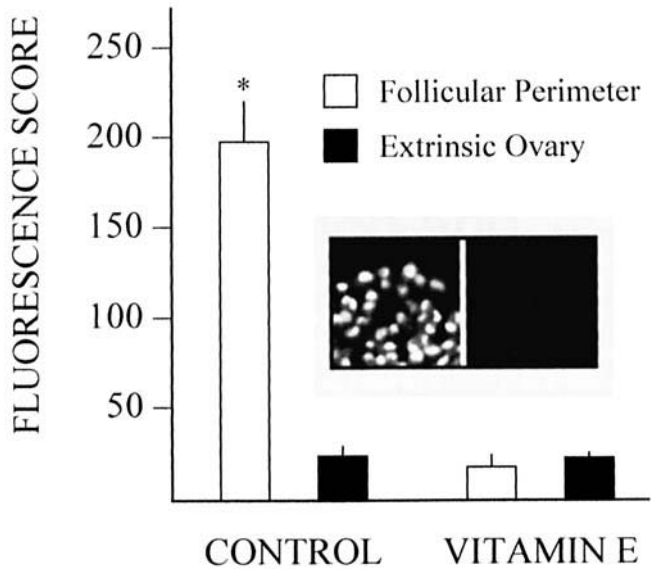

Figure 2. Inhibition by vitamin E of 8-oxoguanine accumulation in sheep ovarian surface epithelial cells recovered from the perimeters of postovulatory follicles. The asterisk indicates a difference ($P < 0.01$) from other means. Shown in the inset are representative photomicrographs depicting fluorescence responses of cells isolated from the perimeter of a ruptured follicle of a control (left panel) and vitamin E-treated (right panel) ewe.

phosphorothioate antisense p53 (5′-CCCTGCTTCCCCTG-GTTC-3′) or sense control (5′-GGAACCAGGGGAAAG-CAGGG-3′) oligonucleotide (Gene Link, Hawthorne, NY). Antisense oligonucleotide was designed to counter a specific region in the ovine mRNA sequence complementary to positions 1164–1183 (45). Cells were evaluated by immunofluorescence for 8-oxoguanine, p53, and CA-125. Conditions used to detect p53 (monoclone KAM-CC002; StressGen Biotechnologies, Victoria, Canada) and CA-125 (monoclone OC-125; Signet Laboratories Inc., Dedham, MA) were the same as those described for 8-oxoguanine; negative controls were performed with primary antibodies preabsorbed with recombinant p53 (Santa Cruz Biotechnology, Santa Cruz, CA) or purified CA-125 (Research Diagnostics Inc., Flanders, NJ).

Functional Capacity and Life Span of the Corpus Luteum in Ewes Treated During the Preovulatory Period with Vitamin E. Ten animals were treated at detection of estrus with vitamin E or vehicle ($n = 5$). Daily jugular blood samples for serum progesterone radioimmunoassay (46) were collected from Day 4 until return to estrus.

Effect of Vitamin E on Pregnancy Outcome in Ewes. Animals treated at detection of estrus with vitamin E ($n = 49$) or vehicle ($n = 50$) were penned with fertile rams. Numbers of lambs born per ewe and lamb health (relative vigor and physical deformities) were recorded.

Statistical Analyses. Assignments to treatments and selections of fields of microscopic inspection were made at random. Subsample data were averaged. Mean comparisons were made by Student's t test or analysis of variance (ANOVA) and protected least significant difference. Serum progesterone profiles were contrasted by split-plot ANOVA. Lambing rates were compared by chi-square.

Results

In comparison to extrinsic sites, levels of 8-oxoguanine were elevated in ovarian surface cells removed from the margins of postovulatory follicles of sheep and humans (Fig. 1).

The increase in 8-oxoguanine in epithelial cells associated with ovulated follicles of ewes was prevented by vitamin E (Fig. 2). Vitamin E had no effect on the preovulatory magnitude of secretion of LH, number of follicular rupture sites, or morphological evidence of luteinization indicative of formation of the corpus luteum (Fig. 3). No oocytes were observed in serial sections of postovulatory follicles, and therefore, expulsion (i.e., ovulation) was assumed.

Concentrations of 8-oxoguanine and p53 in ovarian epithelial cells that were isolated from perimeters of postovulatory follicles of control ewes were greater on Day 2 than Day 6 of control cultures (oxidized bases were deposed by Day 6). Production of p53 by perimetral cells of control animals was inhibited by the antisense oligonucleotide; on Day 6 of culture, 8-oxoguanine adducts prevailed and CA-125 was upregulated. Oxidative damage and tumor suppressor responses to ovulation were subverted in animals treated with vitamin E. Basal concentrations of 8-oxogua-

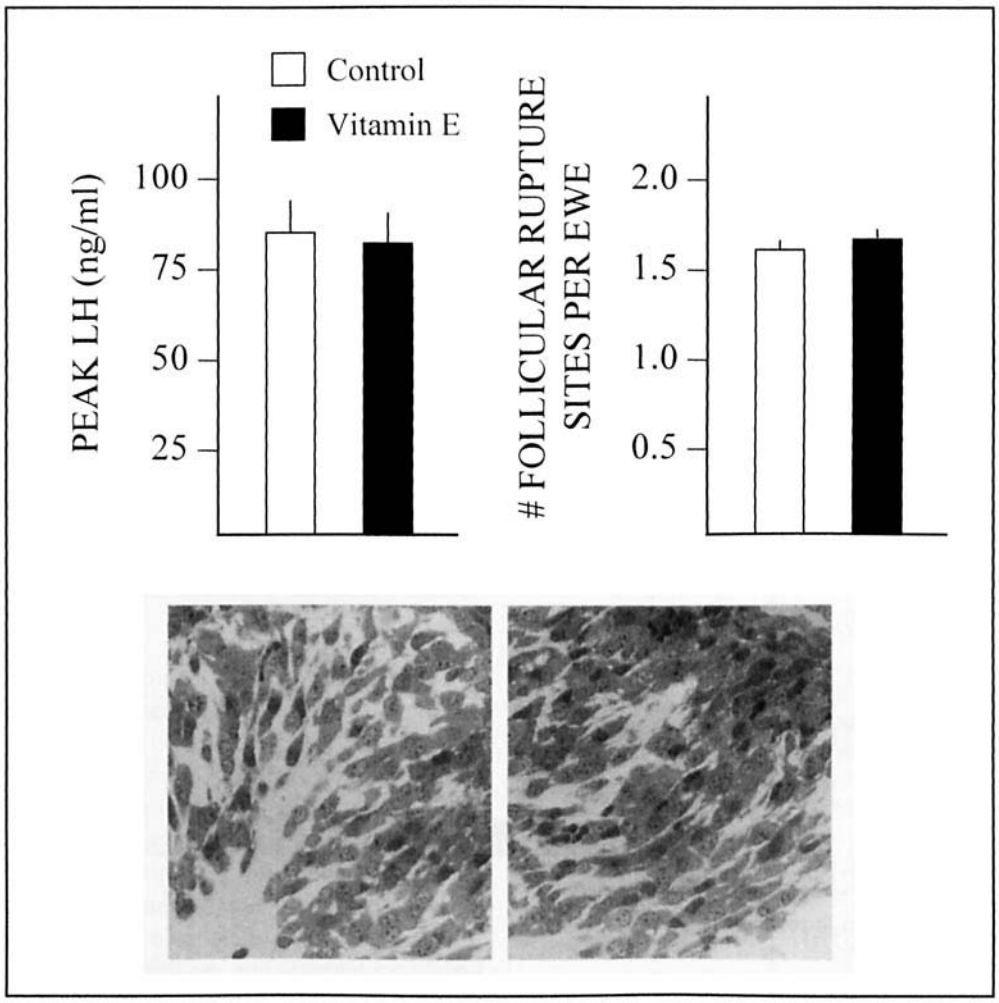

Figure 3. Lack of effects of vitamin E on magnitude of the preovulatory LH peak, follicular rupture, or luteinization. Representative photomicrographs of sections through the follicular wall of a control (left) and vitamin-treated (right) ewe are shown in the lower panel.

nine, p53, and CA-125 in extrinsic cells were not affected by treatments or culture (Fig. 4).

Lengths of estrous cycles (16–17 days) were not altered by vitamin E. Likewise, concentrations of progesterone in jugular serum were unaffected during the post-treatment luteal phase (Fig. 5).

Numbers of ewes that lambed and numbers of lambs per ewe were not different between control (35/50 and 1.3 ± 0.1, respectively) and principal (36/49 and 1.3 ± 0.1, respectively) groups. Furthermore, there was no indication that the health of lambs was influenced by a preovulatory exposure to vitamin E.

Discussion

Base damages to DNA caused by reactive oxygen species are an inevitable by-product of physiological metabolism. To combat this predicament, animals have evolved elaborate enzymatic antioxidant defense mechanisms (superoxide dismutase, glutathione perioxidase, catalase); however, these are less than perfect, and some oxidants find their way to DNA targets (47). Oxoguanine is arguably the most important mutagenic lesion in DNA; mispairing with adenine during replication can cause GC-TA transversions often detected in tumor cells (48, 49).

Results of our initial experiment indicate that 8-oxoguanine distresses to the ovarian epithelium that arise around the time of ovulation in ewes also are manifested in women. A major source of free radicals are those liberated by leukocytes (50) that infiltrate periovulatory follicles (51, 52). Another contributing factor could be the ischemia-reperfusion flux (53) associated with ovulatory follicular rupture and luteinization (51, 54). The ovarian surface epithelium may be vulnerable to genetic damages that are not repaired because it has not been under a strong evolutionary pressure to respond to repeated ovulations (16).

Cultures of sheep ovarian surface cells containing unrepaired DNA exhibited a precursor characteristic (i.e., CA-125 expression) of epithelial carcinoma. CA-125 was first identified over two decades ago as an immunogenic determinant on an ovarian adenocarcinoma cell line (25). The molecular characterization of CA-125 has since proven

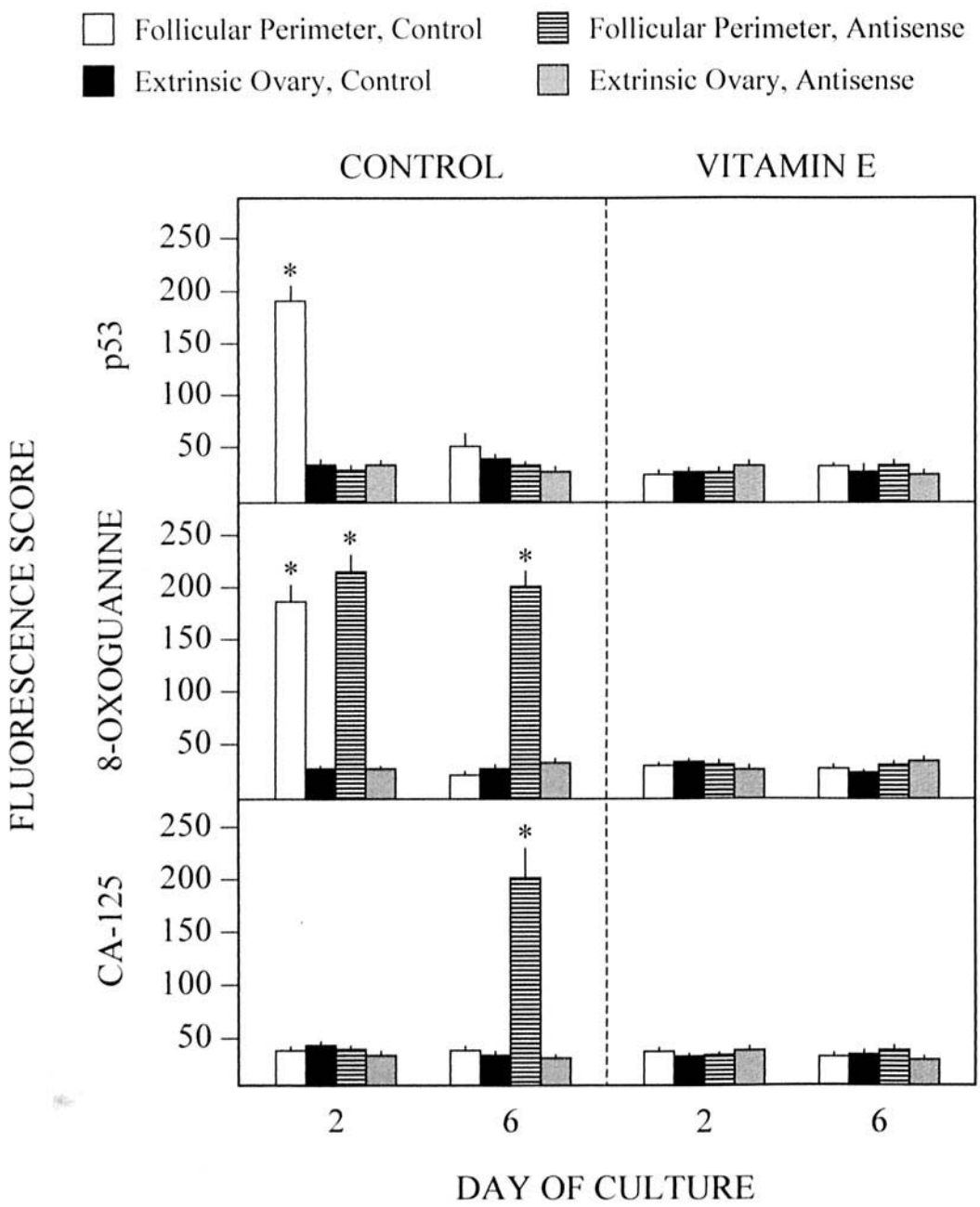

Figure 4. Effects of vitamin E on p53, 8-oxoguanine, and CA-125 immunoreactions in sheep ovarian surface epithelial cells cultured in the absence or presence of a p53 antisense oligonucleotide. Background scores were <28. Asterisks denote increases ($P < 0.01$).

arduous. Most studies have indicated that CA-125 is a heterogeneous high-mass mucinous protein. Size estimates of CA-125 have ranged from 200 to 2000 kd, with smaller subunits being reported (55, 56). The gene encoding CA-125 yields a putative transmembrane molecule with a dominate extracellular motif composed of tandem repeat units, a plasmalemma-spanning domain, and short cytoplasmic tail (57). A pathophysiological function for CA-125 has not been established; roles in tumor cell shedding consistent with early stages of peritoneal spread (55) and in complement inhibition (58) have been proposed.

It is apparent that vitamin E can safeguard the ovarian epithelium from ovulation-induced oxidative DNA damages (and metaplasia). Programmed cellular death within the surface epithelium at the apex of preovulatory follicles, formation of a stigma, and accordingly ovulation are evidently mediated by a differential mechanism (in the sheep, apoptosis is caused by tumor necrosis factor α; Ref. 59) than those provoking oxidative damages to DNA. Ischemia-reperfusion injury to grafts of ovarian tissues was reduced by vitamin E (60). Supplemental vitamin E (e.g., at midcycle) could be of particular value in women considered to be at a preeminent ovarian cancer risk (e.g., those with a genetic predisposition who are not using a contraceptive

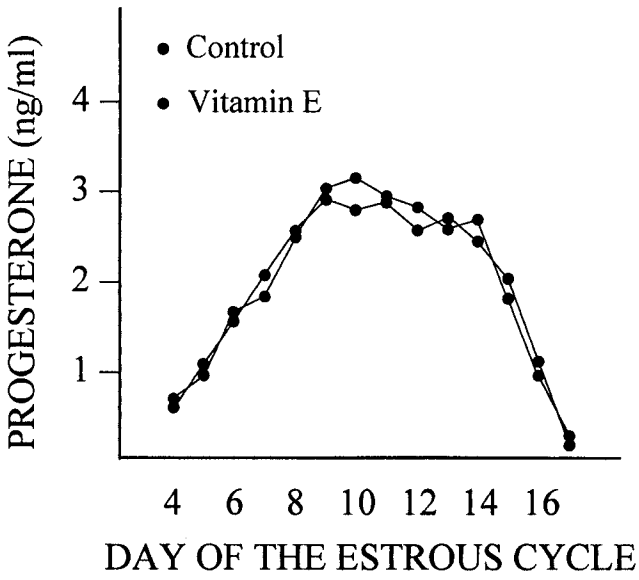

Figure 5. Circulatory progesterone patterns in control and vitamin-treated ewes.

technique that inhibits ovulation). Moreover, it is unlikely that vitamin E would pose a threat in the event of pregnancy.

Vitamin E also can act via mechanisms beyond its oxidant-quenching properties (e.g., by inhibition of protein kinase C and activation of phosphatase 2A and diacylglycerol kinase pathways). Nitric oxide production by endothelial cells and superoxide release by leukocytes is suppressed by vitamin E (61). Nonredox modes of α-tocopherol action include inhibitory and stimulatory effects on rates of mitosis and removal of damaged DNA, respectively (62–65). Therefore, vitamin E could bear secondary (assuming that DNA damage occurred) advantages during the immediate postovulatory period: impeding untoward proliferative responses of ovarian surface epithelial cells until repairs to DNA are accomplished.

The sequences of events that lead to common epithelial ovarian cancer are multifactorial. Several aberrant phases are undoubtedly required to yield a malignant phenotype with distinct growth and metastatic advantages. We suggest that a first step toward tumorigenesis involves oxidative damages to DNA inflicted upon the ovarian surface epithelium as a side-effect of the ovulatory process. Novel findings of this investigation indicate that the antioxidant vitamin E protects ovarian surface epithelial cells by subjugating the deleterious (mutagenic) potential of ovulation.

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