Involvement of Apoptosis and Lysosomal Hydrolase Activity in the Oviducal Regression During Induced Molting in Chickens: A Cytochemical Study for End Labeling of Fragmented DNA and Acid Phosphatase

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ABSTRACT

Induced molting improves egg producing functions in hens. We investigated the mechanism of oviducal regression during induced molting. Involvement of apoptosis and autolysis in the oviducal regression process was analyzed by terminal deoxynucleotidyl transferase (Tdt)-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labeling (TUNEL) and an enzyme histochemistry for acid phosphatase. Nuclei positive for TUNEL were negligible and acid phosphatase staining was weak in the oviduct of laying hens. The frequency of TUNEL-positive nuclei was significantly increased in tubular gland cells of magnum, isthmus, and shell gland 2 d after cessation of egg laying and significantly decreased thereafter. The intensity of acid phosphatase staining was gradually increased during oviducal regression and extremely high on Day 7 after cessation of egg laying. These results suggest that during oviducal regression in induced molting hens, apoptosis is induced in the earlier stage of oviducal regression and autolysis occurs thereafter; eventually, the glandular cells disappear.

(Key words: induced molting, oviducal regression, apoptosis, autolysis, histochemistry)

INTRODUCTION

Molting, a part of the reproduction cycle, is marked by orderly replacement of feathers, and accompanied by total regression of reproductive organs and cessation of egg laying (Johnson, 1986). Induced molting is purported to improve egg producing functions (Nakazawa et al., 1970; Campos and Baiao, 1979; Baker et al., 1981; Lee, 1982) and egg quality (Roland and Bushong, 1979; Garlich et al., 1984; Berry and Brake, 1991; Al-Batshan et al., 1994) in aged laying hens. Cessation of egg laying can be induced by several methods (Wolford, 1984). The most common method for induced molting is feed and water withdrawal, which is combined with or without restriction of light (Nakazawa et al., 1970; Yu and Marquardt, 1973; Wolford, 1984; Decuyperere and Verheyen, 1986). It is assumed that induced molting exerts its effect on egg producing functions by rejuvenation of reproductive organs, including regression and remodeling of oviducal tissues.

The histological process of oviducal regression during the molting period has been examined by previous workers. Eroschenko and Wilson (1974) reported that during the molting period a reduction in cell size of mucosal epithelium and an involution of tubular glands occurred in the oviduct. Quantitatively, Yu and Marquardt (1974) determined that oviduct weight and the number of cells in the magnum, isthmus, and shell gland decreased during oviducal regression. However, the precise mechanism by which oviducal tissues regress remains to be examined.

Two types of cell death, namely apoptosis and autolysis by lysosomal enzymes, are generally accepted to be involved in tissue regression (Bowen and Bowen, 1990). It is possible that these cell death mechanisms are also responsible for the reconstitution process of reproductive organs. Apoptosis plays a role in remodeling of the endometrium during estrus in mammals (Tabibzadeh, 1995). Also, in hens, there are reports suggesting that follicular regression during atresia is accompanied by apoptosis (Tilly et al., 1991; Johnson et al., 1996) and autolysis of follicular cells (Yoshimura and Tamura, 1985; Yoshimura et al., 1989). Apoptosis is a process of physiological cell death and occurs during specific periods of the life cycle of cells. The most specific characteristic of apoptotic cells is the fragmentation of DNA (Gavrieli et al., 1992; Tabibzadeh, 1995). Autolysis by lysosomal hydrolase involves an increase in lysosomal hydrolase activities, which can be detected by an enzyme specific for acid phosphatase (AP) (Alberts et al., 1994). Our goal was to determine the physiological mechanism by which oviduct regression occurs. In this study, we examined the involvement of...
apo... and lyosomal hydrolase activities, respectively, in the oviducal regression during induced molting in chickens.

**MATERIALS AND METHODS**

**Treatment of Birds and Collection of Samples**

White Leghorn hens (approximately 400 d of age) laying two to four eggs in a sequence were kept in individual cages under 14 h light:10 h dark. Molting was induced by feed and water withdrawal. Water was supplied 3 d after the start of withdrawal. Gradual feeding was given from 4 d after cessation of egg laying. The birds were divided into four experimental groups (n = 4 birds in each group); before treatments (L), and 2 d (C2), 7 d (C7), and 14 d (C14) after the cessation of egg laying. Laying hens containing a shell-less egg in the shell gland were examined 6 h after oviposition. Birds were euthanatized by decapitation, and portions of the oviducal tissues (magnum, isthmus, and shell gland) of L, C2, and C7 were fixed with formalin and processed for paraffin sections. The sections (6-µm thick) were air-dried on glass treated with 3-aminopropyltriethoxysilane (Van Prooijen-Knegt et al., 1982) and used to detect the appearance of apoptosis by the terminal deoxynucleotidyl transferase (Tdt-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labeling (TUNEL) method (Gavrieli et al., 1992). Some fresh tissues were embedded in OCT compound and snap frozen in a dry ice-isopentane mixture. Frozen sections were fixed with cold acetone for 30 min, and rinsed in 0.1 M acetate buffer, pH 5.0. The sections were then incubated with reaction mixture at 37 C for 20 min. The reaction mixture consisted of naphtol AS-B1 phosphate as a substrate and pararosaniline as a coupler (Bar... and Anderson, 1962). After incubating, slides were washed with distilled water and counter stained briefly with hematoxylin. Slides were rinsed with running water, dehydrated, cleared, covered, and examined under a light microscope. Control sections were incubated with TUNEL reaction mixture without enzyme (terminal deoxynucleotidyl transferase).

The TUNEL positive nuclei were observed in four different areas in each section (n = 1 for each bird) and their population was analyzed by an image analyzer with a computer system (Mac Aspect, Mitani Co.).

**Acid Phosphatase Detection**

Frozen sections were fixed with cold acetone for 30 min, and rinsed in 0.1 M acetate buffer, pH 5.0. The sections were then incubated with reaction mixture at 37 C for 20 min. The reaction mixture consisted of naphtol AS-B1 phosphate as a substrate and pararosaniline as a coupler (Bar... and Anderson, 1962). After incubating, slides were washed with distilled water and counter stained briefly with hematoxylin. Slides were rinsed with running water, dehydrated, cleared, covered, and examined under a light microscope. Control sections were incubated with reaction mixture without substrate.

**RESULTS**

The oviducal structures, including tubular and mucosal epithelium, were well developed in L and C2, whereas they were markedly regressed in C7 and moderately developed in C14. The average weight of the oviduct was 70.8 ± 1.3, 41.8 ± 3.0, 13.4 ± 1.4, and 45.0 ± 3.6 g in L, C2, C7, and C14, respectively. Figures 1, 2, and 3 show the features of TUNEL-positive nuclei in the magnum of L, C2, and C7, respectively. The TUNEL-positive nuclei were identified by a dark brown stain. The changes in the number of apoptotic cells in the magnum, isthmus, and shell glands during the process of oviducal regression are presented in Table 1. The frequency of TUNEL-positive nuclei in the tubular glands of all oviducal segments was extremely low in laying hens. In contrast, their population was significantly increased in each part of the oviduct in C2. Furthermore, some of the endothelial cells beneath the mucosal epithelium were also positive for TUNEL.
TABLE 1. Changes in the number of TUNEL-positive nuclei in oviducal tubular glands during oviducal regression

<table>
<thead>
<tr>
<th>Group of birds</th>
<th>L³</th>
<th>C2</th>
<th>C7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnum</td>
<td>2.3 ± 0.4³</td>
<td>53.0 ± 5.8²b</td>
<td>13.7 ± 1.1³c</td>
</tr>
<tr>
<td>Isthmus</td>
<td>1.6 ± 0.3³a</td>
<td>20.5 ± 2.1⁰b</td>
<td>9.0 ± 2.0⁰c</td>
</tr>
<tr>
<td>Shell gland</td>
<td>1.4 ± 0.1³a</td>
<td>41.7 ± 4.5³b</td>
<td>8.8 ± 1.8a</td>
</tr>
</tbody>
</table>

Values in a row with no common superscript differ significantly (P < 0.05). TUNEL = Tdt-mediated biotinylated dUTP nick end-labeling. Values are means ± SEM of TUNEL-positive nucleus number in 0.075 mm² (n = 4 birds each). L³ = laying; C2 and C7 = 2 d and 7 d after cessation of egg laying.

(Figure 2) in C2, but not in other hen groups. In the next stage (C7), the number of the TUNEL-positive nuclei in the tubular glands was significantly decreased in each part of the oviduct. The population of TUNEL-positive nuclei was negligible in the mucosal epithelium of magnum, isthmus, and shell gland in L, and this population was not significantly changed in those of C2 and C7 (data not shown).

Table 2 shows the changes in the intensity of AP staining during oviducal regression. The tubular glands of magnum in L exhibited weak AP staining. This intensity slightly increased in C2, and was markedly strong in C7 followed by decreasing in C14 (Table 2 and Figures 4 to 7). In isthmus tubular glands, AP staining was not localized in L, but weak, moderate, and weak staining was observed in C2, C7, and C14, respectively. Changes in the intensity of AP staining in the shell gland tubular gland of L, C2, C7, and C14 were similar to that of magnum tubular gland. Strong AP staining was localized in the mucosal epithelium of magnum of all hen groups (Figures 4 to 7). The isthmus mucosal epithelium of L showed weak AP staining, whereas C2, C7, and C14 areas exhibited moderate staining. Increasing AP staining, which corresponded with oviducal regression, was observed in the mucosal epithelium of the shell gland. In control sections for TUNEL and AP staining, no staining was observed (data not shown).

DISCUSSION

The number of cells showing TUNEL-positive reactions and strong intracellular AP staining was increased in oviducal tissues on Days 2 and 7 after the cessation of egg laying, respectively. The TUNEL-positive cells are thought to be undergoing apoptosis (Gavrieli et al., 1992). Acid phosphatase activity reveals lysosomal enzyme activity because AP is contained in lysosomes (Alberts et al., 1994).

Previous workers have suggested that tubular glands disappear and the mucosal epithelium is diminished in size and height in regressed oviduct (Eroschenko and Wilson, 1974). Also, the first sign of oviducal regression is the disintegration of tubular glands (Gilbert, 1979). In mammals, a significant number of luminal epithelial cells of the endometrium undergo apoptosis when estrogen levels fall (Martin et al., 1973) or RU 486, an antagonist for progesterone, is injected (Slayden et al., 1993). These results suggest that a decrease in estrogen or progesterone levels induces apoptosis in the reproductive tract for remodeling of the tissues. It has been reported that plasma levels of estradiol and progesterone decrease during induced starvation molting (Tanabe et al., 1981) or by injection of gonadotropin-releasing hormone agonist (Dickerman and Bahr, 1989).

TABLE 2. Changes in the intensity of acid phosphatase staining in the oviduct of hens during induced molting

<table>
<thead>
<tr>
<th>Group of birds</th>
<th>L²</th>
<th>C2</th>
<th>C7</th>
<th>C14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnum</td>
<td>+ ± to +</td>
<td>+</td>
<td>+</td>
<td>+ ± to +</td>
</tr>
<tr>
<td>Isthmus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shell gland</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Tubular gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isthmus</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Shell gland</td>
<td></td>
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</tbody>
</table>

Intensities of acid phosphatase reaction product were expressed as − to ++: − = nondetectable, ± = weak, + = moderate, ++ = strong.

L² = laying, C2, C7, and C14 = 2 d, 7 d, and 14 d after cessation of egg laying, respectively.
Immunocytochemistry for steroid hormone receptors have shown that tubular gland cells in the magnum and shell gland of laying hens contain both estrogen and progesterone receptors (Isola et al., 1986; Kusuhara and Ohashi, 1991; Yoshimura and Bahr, 1991; Yoshimura et al., 1995), whereas the mucosal epithelium of these oviducal segments contain progesterone receptor (Isola et al., 1986; Yoshimura and Bahr, 1991), confirming that these cells are the targets for the sex steroids. Therefore, it is possible that an increase in the number of cells undergoing apoptosis (TUNEL-positive cells) in the tubular glands of magnum, isthmus, and shell gland in the initial phase of molting (2 d after cessation of egg laying) may be due to a decrease in sex steroids.

It is generally accepted that lysosomal hydrolases play a role in intracellular turnover in normal cells and for autolysis in degenerating cells (Bowen and Bowen, 1990). The intensity of AP staining increased in the tubular glands of all oviducal segments 7 d after the cessation of egg laying, although the number of TUNEL-positive cells decreased. Therefore, autolysis by lysosomal enzymes may be involved in further deletion of glandular cells after the deletion by apoptosis. Contrarily, the mucosal epithelium of C7 also exhibited increased AP staining. The remaining epithelial cells in the grossly regressed oviduct exhibited diminished height and size (Eroschenko and Wilson, 1974). The increased AP in mucosal epithelium suggests that lysosomal enzymes play a role in the turnover of cytoplasmic components such as secretory substances, resulting in diminished cell size. The AP staining in tubular gland cells and mucosal epithelium was weaker in C14, in which the oviduct was moderately developed in weight and structure, than in C7. This result suggests that lysosomal enzyme is more active in markedly regressing oviduct similar to that of the C7 group.

There are two possibilities for the relationship between apoptosis and autolysis in tubular gland. 1) A decrease in the blood supply as caused by vascular endothelial cell apoptosis as observed in C2 (Figure 2), may affect the glandular cell functions and lead to autolysis. 2) Bowen and Bowen (1990) have suggested that based on genetic hierarchy it might be possible that genes initiating apoptosis may be responsible for triggering the genes responsible for autolytic cascade. Therefore, some of the tubular gland cells may undergo apoptosis followed by autolysis. Because the relationships between apoptosis and autolysis are extremely complex, further studies are required to explain this relationship in the mechanism of oviducal regression.

Collectively, we hypothesize that during induced molting, apoptosis of oviducal tubular gland is induced in the earlier stages of oviducal regression, and that autolysis occurs in the later stages, leading to regression of the tubular glands. Therefore, after induced molting, the oviducal structure becomes histologically similar to that of the immature stage of development, as suggested by Eroschenko and Wilson (1974). It is possible that
when birds initiate egg laying after molting, the oviduct develops new glandular cells, so that function is improved.

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