CHAPTER 6

Regulation of 60K Gelatinase by β-Estradiol*

6.1 Introduction

Different phases of mammary epithelial cell proliferation and differentiation are under stringent hormonal control. Normal mammary gland development is dependent at least in part on hormones such as glucocorticoid [Feng et al., 1995] and estrogen [Ruan et al., 1995]. Hydrocortisone and prolactin [Topper et al., 1975, Ossowski et al., 1979, Topper and Freeman, 1980; Riechmann et al., 1989] inhibited mammary gland regression. Glucocorticoid inhibited involution by a local effect. Since the 60K gelatinase, as described in the previous chapters, appears to have a role in matrix remodeling during involution of the mammary gland, the hormonal regulation of this enzyme in mammary tissue was examined. The effect of β-estradiol which accelerates involution [Athie et al., 1996] of mammary gland, on 60K gelatinase was studied both in vivo and in vitro and the results are presented in this chapter.

6.2 Materials and Methods

Mammary tissues were collected from the 2nd and 6th day involuting glands. In order to study the effect of various hormones on the 60K gelatinase production, epithelial cells were isolated, metabolically labeled in presence of progesterone, prolactin, hydrocortisone, β-estradiol and insulin. The cultures were

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maintained for 18 hrs and the immunoprecipitable radioactivity in the medium was measured.

*In vivo* studies on the effect of β-estradiol was studied by the subcutaneous administration of β-estradiol to lactating rats and subjecting them to involution and collecting the tissue on the 2nd day of involution. Studies on the effect of β-estradiol on cells were monitored by maintaining mammary epithelial cells from 2nd day involuting tissue in primary cultures in presence of β-estradiol. The gelatinase activity was assayed by zymography and quantitated by densitometry.

Synthesis of 60K gelatinase was monitored by metabolic labeling of the isolated mammary epithelial cells from the 2nd day involuting tissue in presence of β-estradiol, followed by immunoprecipitation, SDS-PAGE and quantitation of the radioactivity.

Morphological changes in the hormone treated tissues were analysed by histological examination of the tissue sections. The distribution of the protein was studied by immunocytochemical localisation. Details of the methodology of various experiments are given in Chapter 2.

### 6.3 Results

#### 6.3.1 Effect of different hormones on $^{3}$[H]-60K gelatinase production by mammary epithelial cells

In order to study any possible regulatory effect of hormones on 60K gelatinase, a preliminary experiment was done using primary cultures of mammary epithelial cells. Cells from 2nd day involuting rat mammary gland were isolated for this purpose as the 60K gelatinase was absent and therefore effect of hormones if any, on the production of 60K activity could be detected. Cells in culture were treated with different hormones such as hydrocortisone, prolactin, β-estradiol, progesterone and insulin for 18 hrs. The synthesis of the 60K protein was followed by metabolic labeling with $^{3}$[H]-leucine and immunoprecipitation of the medium with anti-60K gelatinase. Results are given in Fig: 6.1.

Of the different hormones tested, significant increase in the immunoprecipitable radioactivity was observed in cultures treated with β-estradiol and hydrocortisone, when compared to untreated controls. Maximum effect was produced...
Primary cultures of mammary epithelial cells isolated from 2nd day involuting tissue, were treated with $10^{-2}$M each of hydrocortisone (H), progesterone (P), $\beta$-estradiol (E), prolactin (PL) and insulin (I). The cells were metabolically labeled with $^3$H-leucine for 18 hrs. Parallel controls (C) were taken where the system did not contain hormones. The medium was immunoprecipitated with 60K antibody and the radioactivity was measured. The values are the average of 4 assays ± SD.

by $\beta$-estradiol. In presence of prolactin, the immunoprecipitable radioactivity was less than that of control. Although the antibody, as reported before, can precipitate the 90K precursor as well, the results of these preliminary experiments indicated that $\beta$-estradiol and hydrocortisone increased the production of...
60K gelatinase or its precursor. Since the maximum effect was produced by β-estradiol in these experiments, detailed investigations were carried out using β-estradiol.

6.3.2 Effect of β-estradiol on MMPs in vivo

Lactating rats were administered β-estradiol subcutaneously, the pups were removed and subjected to involution. Mammary glands were collected after 48 hrs, processed and subjected to zymography. The activity of different gelatinases are given in Fig: 6.2. Tissues from untreated control showed 130K and 68K gelatinase activity and no 60K gelatinase activity, whereas in hormone treated tissues significant 60K activity was also found indicating that β-estradiol treatment resulted in the production of this enzyme. There was no significant difference in the 68K gelatinase activity in tissues treated with β-estradiol when compared to untreated control. But treatment with β-estradiol caused a marginal decrease (< 15%) though not significant in the 130K activity. 6th day involuting tissue extract also served as a positive control.

6.3.3 Effect of β-estradiol on gelatinase activity in vitro

In order to further examine the effect of hormones on the 60K gelatinase, the production of this activity by mammary epithelial cells isolated from involuting tissues was studied in vitro. Cells isolated from 2nd day involuting tissue were maintained in culture in a serum free medium for 18 hrs in presence and absence of β-estradiol (10^{-4}M). Medium was collected and cells were harvested and subjected to zymographic analysis. Results are given in Fig: 6.3. The hormone treated cultures showed significant 60K activity while this was absent in untreated control indicating that β-estradiol treatment resulted in the induction of 60K activity. Cells from 2nd day involuting tissue produced 130K and 68K gelatinases as well. Absence of any significant effect on other gelatinases in cultures treated with β-estradiol suggested that the stimulatory effect of the hormone on 60K was specific.
Lactating rats were given β-estradiol injections (0.6 mg/kg body wt) subcutaneously and subjected to involution. Saline administered animals served as control. Mammary tissue extracts from the 2nd day involuting tissue of control 2(-) and β-estradiol treated animals 2(+) were subjected to zymographic analysis (upper panel) and compared with the normal untreated 6th day involuting tissue 6(-). The zymograms were quantitated (lower panel). Values given are the average of 5 experiments ± SD.
Fig. 6.3. Effect of β-estradiol on the 60K gelatinase activity in mammary epithelial cells in culture

Mammary epithelial cells from tissue on the 2nd day of involution were maintained in culture for 18 hrs in the presence (+) and the absence of β-estradiol (-). Gelatinase activity in the medium was determined by zymography.

6.3.4 Effect of β-estradiol on the synthesis of 60K gelatinase

The effect of β-estradiol on the 60K gelatinase was further investigated in detail, by studying its biosynthesis by mammary epithelial cells in culture by metabolic labeling experiments. Cultures of mammary epithelial cells from 2nd day involuting tissue were treated with different concentrations of β-estradiol in [3H]leucine containing medium for 18 hrs. Cell pellet and medium were collected, and the radioactive 60K gelatinase was immunoprecipitated by antibody against 60K and quantitated (as described in methods). Results are given in Fig: 6.4.

There was an increase in the amount of [3H]-60K polypeptide in the medium with increase in concentration of β-estradiol. The maximum effect was seen in presence of $10^{-4}$M hormone. β-estradiol effect on the production of 60K-gelatinase was further investigated by treating the cells for different time intervals (2-18 hrs) in presence of hormone ($10^{-4}$M) and the results are given in Fig: 6.5. With increase in time there was a progressive increase in [3H]-60K activity in the medium. In untreated cultures no significant [3H]-60K polypeptide was seen. But certain amount of immunoprecipitable radioactivity could be seen in these controls during early intervals which remained constant throughout the incubation period and may represent the basal level of the precursor of 60K.
Mammary epithelial cells from the 2nd day involuting tissue were metabolically labeled with \( ^{3}\text{H}\)-leucine (25\(\mu\)Ci/ml) in presence of different concentrations of \( ^{\beta}\text{-Estradiol} \) (10\(-7\) to 10\(-2\)M). The \( ^{3}\text{H}\)-60K gelatinase was immunoprecipitated, electrophoresed and quantitated as described in the text. The values given are the average of triplicate assays \( \pm \) SD.

In order to examine if treatment of \( ^{\beta}\text{-Estradiol} \) caused any conversion of the precursor of 60K, the immunoprecipitate of the medium from hormone treated and untreated controls were subjected to electrophoresis and radioactivity associated with the immunoprecipitated proteins was quantified. Results are given in Fig: 6.6. While in the untreated controls there was very little radioactivity associated with 60K protein, significant activity was incorporated into 90K precursor. But in \( ^{\beta}\text{-Estradiol} \) treated cultures apart from \( ^{3}\text{H}\)-90K activity, significant amount of \( ^{3}\text{H}\)-60K was also found. Similarly cells from 6th day involuting
Mammary epithelial cells from the 2nd day involuting tissue were metabolically labeled with $^3$H-leucine (25μCi/ml) in presence of β-estradiol ($10^{-4}$M) for different time intervals. The $^3$H-60K gelatinase was quantitated as in legends to Fig. 6.4. Cultures without β-estradiol were taken as controls (○). The values given are the average of triplicate assays ± SD.

6.3.5 Morphological changes in the mammary gland during normal and induced involution

In order to examine whether the appearance of 60K gelatinase is related to the involutary process, the extent of involution was also studied by looking at the tissue, which produced significant 60K gelatinase, also incorporated significant amount of radioactivity into 60K while no 90K activity could be seen. These results also indicate that the β-estradiol might exert its effect by affecting the mechanism of activation of the precursor.
Mammary epithelial cells from 2nd day involuting gland were metabolically labeled with $^3$H-leucine in presence of $10^{-4}$M $\beta$-estradiol (△). Parallel control were taken where the cells from 2nd (○) and 6th day (●) involuting tissues were metabolically labeled in the absence of hormone. All the cultures were maintained for 18 hrs. The medium was immunoprecipitated with 60K antibody and subjected to electrophoresis. The gels were cut into slices of 10mm thickness and the radioactivity was quantitated as described in methods.

Fig. 6.6. Effect of $\beta$-estradiol on 60K production by mammary epithelial cells

Morphological changes occurring in the mammary gland during normal and induced involution by staining the tissue sections using hematoxylin and eosin (Fig: 6.7.). As indicated earlier the section from virgin tissues showed a flattened epithelial lining with intervening fat tissue. The number and size of the
Fig. 6.7. Morphological changes in the mammary gland on β-estradiol induced involution

Hematoxylin-eosin staining of lactating (A), 2nd day involuting before (B), after (C) β-estradiol treatment and 6th day involuting (D) mammary tissue sections. (→) indicated acini. Bar; 100µm.
acini was minimum. During lactation, the lumen of the acini was dilated and showed evidence of secretion. As involution progressed, on the 2nd day the acini was reduced to about 87% of that of the lactating tissue with slight flattening of the epithelium. On the 6th day of involution the lumen size and width were considerably reduced with the appearance of more intervening fatty tissue indicating involution.

In the hormone treated tissue, the number of acini was reduced to less than 50% of the lactating tissue on the 2nd day of involution while in untreated control the number of the acini was about 85% of the lactating tissue. The size of the acini was also notably reduced in hormone treated tissues. Lumen width was considerably reduced with flattened epithelium. The appearance of fibro-fatty tissue in between the lobules further indicated early involution.

Induction of involution by β-estradiol was also tested biochemically by measuring the amount of casein in hormone treated and untreated control and the results are given in Fig: 6.8. In hormone treated 2nd day involuting tissue significant decrease in the amount of casein was found when compared to untreated control indicating that β-estradiol administration has induced early involution.

6.3.6 Immunocytochemical analysis of the hormone treated tissues

The expression of the 60K gelatinase on treatment with β-estradiol was also tested by immunocytochemical analysis. Sections of lactating, involuting and hormone treated involuting mammary tissues were subjected to immunocytochemical analysis using antibody against 60K gelatinase. Results are shown in Fig: 6.9. Positive reaction was indicated by brown staining. As the involution reached the 6th day, intense staining was seen in the extracellular space indicating the association of the 60K enzyme with the ECM (Fig: 6.9E). A similar pattern was also seen on the 2nd day involuting tissues from animals treated with β-estradiol (Fig: 6.9C). Staining was also seen on the epithelial and myoepithelial cell lining of the acini in normal as well as the estradiol induced involuting tissue. However, the untreated control tissue sections on the 2nd day of involution also showed positive reaction, although less intense, on the epithelial and myoepithelial cell lining of the acini (Fig: 6.9B). Not much staining was seen in
Lactating (L), 2nd day involuting untreated (2I) and after (2IE) β-estradiol administration and 6th day involuting (6I) tissues were subjected to electrophoresis. The amount of casein was quantitated by densitometric analysis and expressed as % of the lactating tissue. Values are the average of four experiments ± SD.

The ECM in the untreated controls when compared to the hormone treated samples. Sections from lactating tissue did not give any positive reaction, further indicating the absence of 60K gelatinase during the lactating phase. Parallel controls of the 2nd and 6th day tissue sections untreated with the primary antibody did not show any staining indicating that the reaction observed was not non-specific.
Fig. 6.9. Immunocytochemical localisation of 60K gelatinase in β-estradiol induced involuting mammary gland

Lactating (A), 2nd day involuting, untreated (B) and β-Estradiol treated (C) and 6th day involuting (E) mammary tissue sections were stained with antibody against 60K and counter stained with Mayers Haematoxylin as described in methods. Tissue sections of the 2nd (D) and 6th (F) involuting day untreated with primary antibody served as control. (→) indicates staining on the periphery of epithelial cells, (←) indicates the myoepithelial staining and (>) indicates the ECM staining. Bar; 100µm.
6.4 Discussion

Results presented above indicate that the 60K gelatinase that is involved in matrix remodeling during involution is subject to hormonal regulation. Evidence in support of this is provided by the observations from in vivo and in vitro studies. (a) Subcutaneous administration of \( \beta \)-estradiol to lactating rats and subjecting them to involution produced 60K gelatinase on the 2nd day while it was absent on the 2nd day of involution in untreated control. (b) Treatment of mammary epithelial cells from 2nd day involuting tissue with \( \beta \)-estradiol also caused the production of 60K gelatinase. (c) Metabolic labeling experiments using mammary epithelial cells from 2nd day involuting tissue in presence of different concentrations of \( \beta \)-estradiol as well as time course analysis showed a concentration and time dependent increase in the incorporation of radioactivity into 60K gelatinase.

The results presented in the previous chapter as well as those described above indicate that the 60K gelatinase, a neutral MMP is involved in matrix remodeling during involution. Evidence in support of this is provided by following observations. (a) The 60K gelatinase activity was observed only at a late stage of involution, but \( \beta \)-estradiol administration led to the production of 60K gelatinase at the early involutary phase. (b) The marked decrease in milk production and the morphological and anatomical changes in the tissue also indicated the onset of early involution due to \( \beta \)-estradiol.

Zymographic analysis and metabolic labeling studies showed that primary cultures of cells from 2nd day involuting tissue did not produce the enzyme. Treatment of these cells with \( \beta \)-estradiol in vitro increased the production of 60K gelatinase. The estradiol effect was concentration dependent and was observed as early as 2 hrs after treatment. The effect of \( \beta \)-estradiol does not appear to be a general effect as 68K and 130K gelatinases were not affected, while only the 60K activity was upregulated in both in vitro and in vivo experiments. In vitro studies also indicate that the hormone exerts a direct effect on the cells in the production of 60K gelatinase and that the effect of \( \beta \)-estradiol observed in vivo is not due to any systemic effect. These results suggest that \( \beta \)-estradiol which promotes mammary gland involution exerts a regulatory role on the 60K
gelatinase which may play an important role in the remodeling of ECM during involutary process by acting on the collagenous components.

Hormones appear to play a key role in regulating MMP activity [Kleinberg et al., 1990] during involution. Hydrocortisone has been shown to specifically inhibit the proteolytic phase and downregulate gelatinase A and stromelysin [Lund et al., 1996] which inhibits involution. Glucocorticoid inhibits involution of the ventral prostate gland after castration and affect apoptosis related gene expression [Rennie et al., 1989, Freeman et al., 1990].

As indicated earlier, immunocytochemical analysis of the 2nd day involuting mammary tissue sections showed brown staining on the epithelial and myoepithelial cell lining whereas on the 6th day of involution intense staining was observed in the ECM as well. In the β-estradiol treated tissues, apart from the staining on the lining of the epithelial and myoepithelial cells, intense staining was observed in the ECM as well, indicating the presence of 60K gelatinase. As described in the previous chapter, the 60K gelatinase is produced as a 90K inactive precursor. The presence of such an inactive precursor may explain the positive staining observed in immunocytochemical analysis of the untreated control tissues on 2nd day of involution and is associated with the epithelial and myoepithelial cells and not in the ECM. It is not clear whether estradiol affects the activation of the precursor.

The enzyme 60K gelatinase appears to be crucial in involution as preliminary in vitro experiments with prolactin caused reduction in immunoprecipitable radioactivity. Lactation is induced in response to prolactin, as the prolactin; effect is reduced and the estrogen stress increases, lactation stops and the gland progressively undergoes involution. But it is not clear how β-estradiol regulates the activity. Results reported in the previous chapter indicate that 60K gelatinase is produced as a 90K proenzyme and the precursor is secreted into the extracellular space and gets activated. On the 2nd day of involution, although no 60K activity was present, immunoblot and immunoprecipitation showed cross reaction due to the presence of the 90K precursor. Metabolic labeling of cells from 2nd day involuting tissue in presence of β-estradiol caused the production of 60K gelatinase. Immunoprecipitation and analysis of the medium showed that significantly high amount of radioactivity was associated with the 60K protein
than the 90K, whereas in untreated control the radioactivity associated with 60K was very low compared to the precursor. This indicated that β-estradiol may affect the activation of 90K precursor to 60K gelatinase. This indicates that one of the molecular mechanisms involved in induction of involution of mammary gland may be through its effect on MMPs, particularly 60K gelatinases which can degrade collagen of ECM. Further detailed investigations are required to establish the exact mechanism of action of β-estradiol in this process.