INVOlVEMENT OF GLUCOCORTICOID IN INSULIN-INDUCED ANGIoGENESIS OF ADJUVANT POUCH GRANULOMA IN DIABETIC MICE

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The effects of insulin and glucocorticoid on granuloma formation and angiogenesis were studied using an adjuvant pouch in mice and a carrageenin pouch in rats. Carrageenin-induced granuloma formation was suppressed in the diabetic state induced by alloxan in rats. The suppression was restored by adrenalectomy. Corticosterone counteracted the restoration, whereas epinephrine did not, suggesting that the effects of adrenalectomy are due to the lack of adrenocortical hormones rather than epinephrine. The blood corticosterone levels in alloxan mice increased, following the increase of glucose level, to more than 20 μg/dl after 4 weeks. The increase of corticosterone levels disappeared after adrenalectomy. In the mouse adjuvant pouch, corticosterone dose-dependently decreased granuloma formation and angiogenesis. The values of these two parameters obtained with a dose of 20 μg/pouch corticosterone agreed with those of the serum levels in diabetic mice. Insulin dose-dependently reversed the suppressed angiogenesis of 20 μg corticosterone-treated mice and the dose-response curve approximated the curve of alloxan-treated mice. The effects of diabetes were concluded to involve insulin deficiency as well as glucocorticoid enhancement and the counteraction between the two may control granuloma formation and angiogenesis.

Keywords — glucocorticoid; angiogenesis; granuloma; diabetic mouse; KK-CAy mouse; adrenalectomy; insulin-induced angiogenesis; corticosterone-decreased angiogenesis

INTRODUCTION

Adjuvant pouch granuloma formation has been demonstrated to be suppressed by the diabetic state and restored by the administration of insulin in alloxan-induced diabetic mice, an insulin-deficient model.¹ At the concentration of insulin used, however, the insulin effect did not decrease the blood glucose levels of diabetic mice, indicating dependence on the growth factor induced by insulin. In non-insulin dependent diabetic mice (KK-CAy), insulin resistance caused a decrease in granuloma formation.² The insulin resistance in the pouch granuloma is postulated to be caused by any antagonistic mediator induced by inflammation rather than by the inactivation of insulin receptors.

Because of the insulin antagonistic mediator in inflammation, the present study focused on corticosterone. Corticosteroids are reported to be another factor involved in the regulation of granuloma formation.³ We also confirmed the effect of hydrocortisone on the exudate volume and the granuloma weight of adjuvant in mice.⁴ Blood corticosterone levels are reported to increase in the diabetic state,⁵ suggesting that corticosterone is possibly involved in the decreased granuloma formation in diabetes. Other investigations have subsequently shown that glucocorticoid treatment or hyperadrenocorticism results in insulin resistance.⁶⁷

The aim of the present study was to reveal the interaction between insulin and glucocorticoid in the angiogenesis of adjuvant pouch granuloma in diabetic mice because angiogenesis of the granuloma plays an intimate role in proliferative inflammation¹ and has a high sensitivity of insulin in diabetic mice.⁸

MATERIALS AND METHODS

Animals — Six-week-old ddY mice (male) and Wistar rats (female), weighing 200 g, were obtained from the Shizuoka Laboratory Animal Center (Hamamatsu). Diabetic mice and rats were produced by a single injection of alloxan monohydrate, 85 mg/kg, i.v. and 175 mg/kg, i.p., respectively.

Mouse Adjuvant Pouch Method⁴ — An air pouch was produced by subcutaneous injection of 3 ml air into the back of each mouse. After 24 h, a phlogistic agent, Freund's complete adjuvant

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containing 0.1% croton oil (0.5 ml), was injected into the air pouch. Test drugs were administered into the air pouch just after the adjuvant and the administration was repeated once every day until the day before the animal was sacrificed. Some mice were pretreated with alloxan 7 d before injection of the adjuvant.

**Rat Carrageenin Pouch Method** — Rat carrageenin pouch was prepared according to the method of Tsurufuji et al. Seven milliliters of air were subcutaneously injected into the back of each rat. After 24 h, 4 ml sterilized carrageenin solution (2% w/v) previously warmed to 40 °C, were injected into the air pouch. Some of the rats were pretreated with alloxan 8 d before and adrenalectomized 5 d before the carrageenin injection.

**Quantitative Measurement of Newly Formed Blood Vessels (Carmine Dye Method)** — One milliliter of carmine dye suspension (10% w/v) in 5% gelatin solution was injected into the tail vein of each mouse with an adjuvant granuloma pouch. After the gelatin was solidified by cooling the cadaver below 4 °C, the granuloma tissue was removed. The tissue for each mouse was dissolved in 6 ml 3 N NaOH solution for exactly 40 min at room temperature and then acidified with 3 ml concentrated HCl, since carmine dye is labile in alkaline solution but stable in acidic solution. The digested samples were centrifuged at 2500 rpm for 20 min and then the supernatant fluid was filtered through Millipore filters (0.45 μm). The filtrates were measured spectrophotometrically at 530 nm against a blank tissue specimen obtained without the infused carmine dye.

**Measurement of Blood Corticosterone Levels** — Blood was obtained from normal and adrenalectomized mice following decapitication. The collected blood samples were allowed to settle for 20 min at room temperature and then the serum samples were separated by centrifugation at 2500 rpm and stored at −85 °C until assay.

Serum corticosterone levels were measured fluorometrically by the method of Mejer and Blanchard with minor modifications. Each 0.5 ml serum or standard corticosterone was added to a 20-ml stoppered test tube containing distilled water (1.5 ml), 0.5 N NaOH solution (0.2 ml) and methylene chloride (10 ml). The mixture was then shaken 100 times. After the solution stood for a few minutes, the water phase was aspirated and the methylene chloride phase was washed with 1 ml distilled water, which was also aspirated. In another test tube, 7 ml methylene chloride were added to 3.5 ml cold fluorescence reagent (ethanol 3: sulfuric acid 7). After the test tube had been shaken 100 times, the upper phase (methylene chloride) was removed by aspiration. The fluorescence intensity of the acidic layer (lower phase) was then measured within 40 to 60 min after the addition of a fluorescence reagent at 470 nm (excitation) and 510 nm (emission) wavelengths. The standard curve was linearly related to the concentration of corticosterone in the range of 0—30 μg/ml.

**Materials** — Alloxan monohydrate, Arlacel-A (mannide monooleate), croton oil (Nakarai Chemicals), liquid paraffin, carmine dye (Merk), methylene chloride (Dotite Luminal®), corticosterone, insulin (Sigma), carrageenin (Seakem #202, Marine colloid Inc.), and a killed preparation of *Mycobacterium tuberculosis* (Aoyama-B strain) were used.

**Statistical Evaluation** — The data obtained were analyzed by the unpaired *t* test and the criterion for statistical significance was *p* = 0.05 level.

**RESULTS**

**Effects of Adrenalectomy and Adrenal Hormones on Pouch Granuloma Formation Induced by Carrageenin in Diabetic Rats**

Pouch granuloma formation was induced by 2% carrageenin in diabetic rats after 5 d when administered 175 mg/kg alloxan monohydrate. Granuloma weight was measured at 7 d after carrageenin treatment. As shown in Table I, granuloma weight decreased significantly in diabetic rats as compared with normal rats, and then the decrease of formation of granuloma tissue was restored by bilateral adrenalectomy of diabetic rats.

In adrenalectomized rats, the effect of adrenal hormone was determined by the injection of 1.0 mg/kg corticosterone and 0.1 mg/kg epinephrine into the pouch once each day after the day of carrageenin treatment. The dose ratio of corticosterone and epinephrine used was 10 : 1, which was the ratio in blood concentrations between the two hormones. Although corticosterone significantly decreased the wet weight of granuloma, epinephrine did not affect it. Epinephrine, however, had a tendency to increase the wet weight when combined in the same dose with corticos-
TABLE I. Effect of Corticosterone (CS) and Epinephrine (Ep) with Adrenalectomy (Adrex) on Granuloma Formation in Alloxanized Diabetic Rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Treatment</th>
<th>(n)</th>
<th>Granuloma wet wt. (g)</th>
</tr>
</thead>
</table>
| Normal   | Sham op.        | (10)| 5.881 ± 0.301
| Diabetics| Sham op.        | (6) | 4.685 ± 0.437
|          | Adrex           | (4) | 5.900 ± 0.642
|          | Adrex + CS (1 mg/kg) | (5) | 3.912 ± 0.134
|          | Adrex + Ep (0.1 mg/kg) | (4) | 5.909 ± 0.371
|          | Adrex + CS + Ep | (6) | 4.888 ± 0.597

a) Mean ± S.E. of 4 - 10 rats. b) p < 0.01. NS; not significant (p = 0.05).

TABLE II. Time-Course of Serum Corticosterone Levels after Alloxan Treatment in Mice

<table>
<thead>
<tr>
<th>Weeks after treatment</th>
<th>(n)</th>
<th>Serum corticosterone (μg/dl)</th>
<th>Alloxan treated</th>
<th>Age-matched control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(9)</td>
<td>11.50 ± 1.19</td>
<td>4.39 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(9)</td>
<td>13.62 ± 1.66</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(9)</td>
<td>18.06 ± 3.19</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(8)</td>
<td>24.27 ± 3.22</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(9)</td>
<td>22.13 ± 4.00</td>
<td>3.68 ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

a) Mean ± S.E. b) Not determined.

Corticosterone, compared with the effect of corticosterone alone.

Blood Corticosterone Levels in Diabetic Mice

Whether the adrenocortical hormone involved in the decrease of granuloma formation in the diabetic state was ascertained by measuring the blood corticosterone level. Alloxan administration increased the blood glucose levels after 24 h and attained a maximal level at 48 h, whereas the blood corticosterone levels began to increase after 3 d (Fig. 1). The serum corticosterone gradually increased about threefold after one week and sevenfold after 5 weeks, in parallel with the length of time for the induction of diabetes (Table II). The effect of alloxan was confirmed by comparing corticosterone concentrations in adrenalectomized mice to those of normal mice. Serum corticosterone and glucose levels were measured 3 weeks after treatment with alloxan in adrenalectomized mice. As shown in Table III, adrenalectomy inhibited the alloxan-induced increase of blood corticosterone levels with no changes in the blood glucose levels. An averaged level of 1.78 μg/dl serum corticosterone in adrenalectomized normal mice was considered as non-specific serum fluorescence.

Effects of Insulin and Corticosterone on Granuloma Formation and Angiogenesis

Corticosterone administration reduced granuloma formation and angiogenesis in a dose-
TABLE III. Effect of Alloxan on Corticosterone Concentrations in Normal and Adrenalectomized Mice

<table>
<thead>
<tr>
<th></th>
<th>(n)</th>
<th>Serum corticosterone (µg/dl)</th>
<th>Serum glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>(10)</td>
<td>4.39 ± 0.49</td>
<td>138.8 ± 5.3</td>
</tr>
<tr>
<td>Alloxan</td>
<td>(9)</td>
<td>18.06 ± 3.19</td>
<td>582.0 ± 14.2</td>
</tr>
<tr>
<td>Adrenalectomy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>(10)</td>
<td>1.78 ± 0.27</td>
<td>150.6 ± 6.0</td>
</tr>
<tr>
<td>Alloxan</td>
<td>(6)</td>
<td>2.75 ± 0.37</td>
<td>567.2 ± 28.5</td>
</tr>
</tbody>
</table>

Number: mean ± S.E. of 6—10 mice, measured 3 weeks after adrenalectomy.

FIG. 2. Dose-Response Relationship between the Concentration of Corticosterone and Granuloma (●) or Blood Vessel (□) Formation

Corticosterone was injected into the pouch. Note that corticosterone dose-dependently decreased granuloma formation and angiogenesis. The effect of 20 µg corticosterone was similar to that of diabetic state.

FIG. 3. Effect of Insulin on Pouch Angiogenesis in the Presence of Corticosterone in Normal Mice (●) and in the Absence of It in Diabetic Mice (○)

Drugs were injected into the pouch daily. Note that the effect of insulin in the presence of 20 µg corticosterone was similar to the effect on diabetic mice.

dependent manner (20—80 µg/pouch). The values of granuloma formation and angiogenesis induced by 20 µg corticosterone in the dose-response curves were very similar to the values of granuloma formation and angiogenesis induced in diabetic mice shown in Fig. 2. The results suggested that the blood corticosterone levels were increased to approximately 20 µg/dl in the diabetic state.

To determine the relation between the effects of corticosterone and insulin on the decreases in angiogenesis, the dose-response curves of insulin were investigated for each dose of corticosterone (20—80 µg/pouch). When the percentages of increased angiogenesis were calculated on the basis of values observed in the absence of insulin for each dose of corticosterone, they were dose-dependently potentiated by insulin (10—50 U/kg, i.p.) (Fig. 3). The dose-response curve with 20 µg corticosterone approximated with the curve observed in diabetic mice. At a high dose (80 µg/pouch) of corticosterone, insulin failed to potentiate angiogenesis.

DISCUSSION

Diabetic state-induced decrease of granuloma formation and angiogenesis have previously been reported in alloxan-induced diabetic mice\(^1\) and genetically inbred diabetic KK-CA\(^y\) mice.\(^2\) These effects are restored reversibly by insulin treatment. The decrease is considered to
be due to a lack of insulin or to insulin resistance for a relative deficiency of action of insulin. As previously discussed, the action of insulin in these restoration was not considered to be based on the lowering of blood glucose levels. In the diabetic state, however, hypothalamo-pituitary adrenocortical system activity has been reported to increase. The present study also demonstrated not only an increase in blood corticosterone levels in diabetic mice but also a counteraction between corticosterone and insulin.

The blood corticosterone levels increased in diabetic state was confirmed by adrenalectomy. The serum corticosterone was involved in diabetic state rather than the serum epinephrine. The serum corticosterone increased few days after an increase in the blood glucose level. This suggested that the increase of serum glucose or the decrease of serum insulin may initiate the secretion of corticosterone. The diabetic state-induced increase in serum corticosterone was more than 20 μg/dl at the 4th week after treatment with alloxan and about sevenfold for an age-matched control after 5 weeks. The effects of adrenalectomy completely eliminated the increase but did not change the blood glucose levels. This indicated that the diabetic state-induced suppression of pouch granuloma formation depended on the effect of corticosterone but not on the blood glucose levels.

The effect of insulin is considered to depend directly on a cell growth factor including angiogenesis. The mechanism of insulin action was postulated as the counteraction against corticosterone, suppressing the angiogenesis of pouch granuloma. In alloxan-induced diabetic mice, the pouch granuloma was about 0.3 g wet weight and carmine in the blood vessel was about 0.2 mg. These values agree with those induced by the injection of 20 μg/pouch corticosterone, when estimated from the dose-response curve of corticosterone. This indicated a dose balance of counteraction between the corticosterone increased and insulin decreased by the diabetic state. This was supported by the results showing the dose-response curve of insulin for angiogenesis in diabetic mice agreed with that of insulin in the presence of 20 μg corticosterone. When the dose of corticosterone was increased, however, the dose-response curve of insulin was noncompetitively inhibited by corticosterone. This suggested that the site of action of insulin is not corticosterone receptor for counteraction.

The mechanism by which insulin induces the angiogenesis of granuloma seems to act directly on the neogenesis of blood vessels independent of corticosterone receptor. Because it has been reported that chronically treated glucocorticoid decreased the insulin binding, streptozotocin-induced diabetes did not change either the number or the affinity of glucocorticoid receptor. Since glucocorticoids inhibit the effects of insulin which requires gene expression, serum glucocorticoid may affect the post-process in insulin receptor mechanisms. In a recent hypothesis concerning the molecular mechanism of glucocorticoid action, the receptor is suggested to be a hormone-activated switch required for the activation of glucocorticoid-dependent cellular enhancement. Therefore, the insulin action for growth factor may be concluded to explain the counteraction against the regulatory function of cell to corticosterone.

REFERENCES
10) L. E. Mejer and R. C. Blanchard: Fluorometric determi-


