Tumour Specific and Immunosuppressive Components in Soluble Cell Extracts from Ovine Squamous Cell Carcinoma

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SUMMARY

The ovine squamous cell carcinoma (OSCC)-specific and immunosuppressive properties of OSCC extracts were investigated by using the techniques of lymphocyte blastogenicity, acid dissociation-ultrafiltration and gradient polyacrylamide gel electrophoresis. It was found that OSCC extracts contained two major and one minor protein peaks by Sephadex gel fractionation. Two major peaks bear substantial amount of immunoglobulins, antigen-antibody complex and OSCC-specific fractions, and the minor peak includes immunosuppressive materials. OSCC-specific components were detected at the molecular weights of 10,000 to 100,000 daltons in the major peaks and immunosuppressive materials at the fractions with the molecular weight of 10,000 to 100,000 and <10,000 daltons in the minor peak. When the fractions were further separated by gradient polyacrylamide gel electrophoresis, the OSCC-specific antigens were found in the slice number 4 to 6 in fraction III, and immunosuppressive materials, in the slice numbers 9 to 11 in fraction V.

The present results were considered to provide a basis for preparation and purification of OSCC-specific and immunosuppressive materials from the crude OSCC extracts.

INTRODUCTION

Crude 3M KCl extracts of ovine squamous cell carcinoma (OSCC) have shown the ability to enhance tumour growth and metastatic spread by a single intramuscular injection of OSCC protein to tumour-bearing sheep\textsuperscript{11}. Such extracts also induce blastogenesis of lymphocytes from OSCC-committed, or, to some extent, normal adult sheep\textsuperscript{8,9,10,12}. Recent works\textsuperscript{7} reported that a fraction of crude OSCC extracts separated by Sephadex gel fractionation caused in vivo immunosuppressive

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to normal sheep. In contrast, crude 3M KCl extracts of normal sheep skin have shown no similar associations with tumour cell-mediated immune reactions.

The present communication describes attempts to identify and characterise OSCC-specific and immunosuppressive components in the crude 3M KCl extracts of OSCC in comparison with ovine normal skin extracts.

MATERIALS AND METHODS

1. Water soluble cell extracts

Two OSCC(a,b) amputated from ear-located stage III lesions and 2 normal tissues from sheep ear skin(c,d) were used to prepare water soluble extracts by 3M KCl extraction technique described by Jun et al\(^{11}\) and Brandchaft et al\(^{22}\).

2. Sephadex gel fractionation

Forty ml of soluble extracts from OSCC and normal tissues at the protein concentration of 4.5–4.8 mg/ml was fractionated through Sephadex G-150 column as described by Jun\(^{7}\). For in vitro assays, fractions were sterilised by filtration through a 450 nm millipore filter.

3. Lymphocyte blastogenicity assay

Sources of lymphocytes;

Five healthy sheep and 3 OSCC-bearing sheep, aged 3 years, were obtained from one flock of Peppine Merino and used as normal(NL) and OSCC-sensitized (OSL) lymphocyte donors, respectively. OSL was prepared as described by Jun and Johnson\(^{8,10}\).

Assay procedures;

A standardised micromethod described by Jun et al\(^{12}\) was used throughout tests. NL and OSL were cultured with fractionated proteins alone, or fractionated proteins plus phytohaemagglutinin-p (PHA-P). When PHA-P was added in culture, the microplates with lymphocytes and test fractions (40 μl/culture) were incubated at 37°C for 1 hr in a humidified 5 % CO\(_2\)-95 % atmosphere, before addition of PHA-P(1 μg/200 μl).

Results were reported as mean count per minute (cpm) of stimulated cultures subtracted mean cpm of unstimulated controls.

One-way mixed lymphocyte culture (MLC) was also applied with a slight modification of the method described by Jun\(^{7}\). Briefly, 40 μl of fractions was added to the microwells with responder cells (2 × 10\(^5\) cell/100 μl), and the plate was incubated at 37°C for 1 hr. A mitomycin-treated stimulator cells (2 × 10\(^5\) cells/100 μl) was then added to microwells. Results were recorded as mean cpm of cultures.

Data from independent samples were analysed by the Student’s test, and data from paired samples by a paired t test. Differences between comparisons were considered significant if p<0.01\(^{7}\).

4. Immunoelectrophoresis (IE)

A modification of the standardized micro-method described by Ouchterlony and Nilsson\(^{23}\) was used for immunoelectrophoretic analysis of the fractionated proteins against rabbit anti-sheep serum.

5. Acid dissociation and ultrafiltration

A modification of the method described by Sjögren et al\(^{29}\) and Murray et al\(^{21}\) were used to dissociate immunoglobulin molecules in Sephadex fractions. Briefly, fractions (1 ml) were diluted 1 : 20 with Sorensen’s glycine buffer, pH 2.8, and introduced into an Amicon Diaflo cell with an XM-100A membrane, retaining the >100,000 daltons (E100). Filtration being carried out at 25 p.s.i., 20 ml buffer was further added dropwise. Retentate including immunoglobulin molecules was collected at 2 ml volume, and both solutions were adjusted to pH 7.1 with 7.5 % NaHCO\(_3\). The filtrate excluding immunoglobulin molecules was then passed through a PM-10 membrane, separating the retentate of 10,000 to
100,000 daltons (E 10-100) and the filtrate of <10,000 daltons (E10). All fractions were dialysed against phosphate buffered saline, pH 7.1 (PBS), concentrating into 1 ml volume, and sterilised by filtration.

6. Gradient gel electrophoresis

For further identification of active protein components in extracts, gradient polyacrylamide gel electrophoresis was carried out with a modification of the method of Margolis and Kendrick. Two ml of fraction from Sephadex column fractionation was applied to polyacrylamide gels (82 x 70 mm) in layers of 4 to 24 % prepared in 0.05 M glycine buffer, pH 10, and subjected to electrophoresis for 24 hrs at 80 V, 60 mA in a Gradiapore electrophoresis bath. Gels were sliced across the direction of electrophoresis into 14 strips by 0.5 cm wide, and each strip was disrupted by forcing through syringe, then passively eluted into 2 ml of 0.05 M glycine buffer, pH 10, for 24 hrs. For in vitro assays, the eluates of slices were adjusted to pH 7.1 by addition of 1 N HCl, and sterilised by filtration through a 450 nm millipore filter.

Protein measurement of all fractions was carried out as described by Miller.

RESULTS

1. Sephadex fractionation of extracts

Figs. 1A and 1B illustrate the profile of Sephadex G-150 column fractionation of 2 OSCC extracts and 2 normal tissue extracts. OSCC extracts revealed 2 major and 1 minor peaks with the similar pattern to normal skin tissue extracts. Five fractions of OSCC and normal tissue extracts were prepared by pooling the fractions as indicated in Figs. 1A and 1B, according to the similar molecular sizes. After concentrating the fractions by ten times, the contents of

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Fig. 1. Elution profile of Sephadex G-150 column chromatography of 2 OSCC (A) and 2 normal skin (B) extracts. Five fraction of different molecular size were obtained in both extracts by pooling the fractions as indicated. Immunoglobulins in the fractions concentrated by one-tenth were tested by immunoelectrophoresis against rabbit anti-sheep serum. ---; IgG, ——; IgM.
immunoglobulins were analysed by immunoelectrophoresis. As shown in Figs. 1A and 1B, fraction I of OSCC revealed strongly positive reaction to Ig G and trace to Ig M, and fraction II, III of OSCC extracts and fraction i, ii of normal tissue extracts, moderately positive or trace reactions to Ig G. Fraction IV, V of OSCC and fraction iii, iv, v of normal tissue extracts showed no reaction in immunoglobulin regions.

2. Effects of Sephadex-fractionated extracts on lymphocyte blastogenicity

The Sephadex fractions obtained from the extracts of OSCC and normal tissue were tested for blastogenesis to OSL and NL. The results are illustrated in Figs. 2A and 2B. OSCC fractions, No 47 to 62 at fraction III region, showed the highest stimulation of OSL and slight (p<0.01) stimulation with NL. The response at peak was higher (p<0.002) than that of the unfractionated OSCC extracts at the same protein concentration. No 32 to 41 at fraction I region of the OSCC extracts revealed lower response to OSL than the fractions from fraction III region, but very significant stimulation (p<0.001), compared to PBS control. OSCC fractions, No 75 to 86 in fraction V region, induced significant suppression (p<0.01) of lymphocyte blastogenicity with both OSL and NL, compared to PBS control (Fig. 2A).

Fractions from normal tissue extracts induced no stimulation of NL, but slight stimulation was shown (p<0.01) in OSL at the fractions No 53 to 60 of fraction iii.

Specificity of the fraction No 56 of normal tissue extracts was tested by comparison with the fraction No 53 of OSCC extract. As shown in Fig. 3, OSCC fraction No 53 showed consistent increase of OSL blastogenicity according to in-

![Diagram](image-url)

Fig. 2. Effects of OSCC (A) and normal tissue (B) extracts fractionated by Sephadex G-150 column chromatography on blastogenicity of OSCC-sensitised (○) and normal (●) lymphocytes. Protein concentration of fractions were adjusted by 5μg per 200μl culture. Asterisks represent the mean cpm ± SD of unfractionated OSCC and normal tissue extracts against OSCC-sensitized lymphocyte, respectively. The mean cpm of PBS control to both lymphocytes = 1,785 ± 297. Each point represents mean cpm of lymphocytes from 3 different animals in triplicate cultures.
creasing concentration of protein, whereas normal tissue fraction No 56 revealed no such increase of OSL blastogenicity. This discrepancy implies that the transient stimulation of OSL with normal tissue extract at fraction iii (Fig. 2B) is non-specific reaction to OSCC.

Five pooled fractions of OSCC and normal tissue extracts were tested for effects on PHA-induced blastogenicity to OSL and NL. As illustrated in Figs. 4A and 4B, fractions IV and V of OSCC extracts induced a significant suppression of PHA-induced blastogenicity of OSL and NL (OSL, p<0.001; NL, p<0.01). The suppressive effects appeared more evidently with OSL than NL (Fig. 4A). The fractions of normal tissue extracts showed no significant changes of blastogenicity in both OSL and NL (Fig. 4B).

Effects of the fractions on lymphocyte blastogenicity were also tested by one-way mixed lymphocyte culture. As shown in Figs. 5A and 5B, fraction V of OSCC extracts induced very significant suppression (p<0.001) in MLC in both combinations of stimulator vs responder (Dm X E and Dm x F). Fraction III of OSCC caused a slight stimulation in MLC, compared to PBS control (Fig. 5A). Normal tissue fractions showed no significant changes (Fig. 5B).

3. Effects of the acid dissociated subfractions on lymphocyte blastogenicity

Subfractions obtained by acid dissociation and ultrafiltration of the Sephadex fractions were tested for effects on lymphocyte blastogenicity to characterise further the OSCC specific and immunosuppressive components of the OSCC extracts (Table 1). When the subfractions were cultured with NL, without addition of PHA-P, increased blastogenicity was observed at the subfraction E10 - 100 of OSCC fraction III. Subfraction E10 of fraction V induced suppressive blastogenicity of NL (p<0.001). When the subfractions were cultured with OSL, without addition of PHA-P, significantly increased blastogenicity was revealed in the subfraction E10 - 100 of OSCC fraction I, II, III and IV, showing the maximal increase (p<0.001) at E10 - 100 of OSCC fraction III. E10 - 100 of OSCC fraction showed higher stimulation with OSL (p<0.01), compared to maximum response with undissociated fractions in fraction I (Fig. 2A). Both subfractions of E10 - 100 and E10 of OSCC fraction V induced suppressive blastogenicity of OSL.

When NL and OSL were cultured with subfractions plus PHA-P, no changes were observed at any subfractions except E10 - 100 and E10 of OSCC fraction V that induced very significant (p<0.01, p<0.001) suppressive blastogenicity of both NL and OSL.

4. Effects of fractions separated by gradient
Fig. 4. Effect of five fractions of OSCC(A) and normal tissue (B) extracts from Sephadex gel column fractionation on PHA-P induced blastogenicity of OSCC-sensitised (○) and normal (●) sheep lymphocytes. Protein concentration of fractions is 5μg per 200μl culture. Each point represents mean cpm ± SD of lymphocytes from 3 different sources in triplicate cultures. Significance of differences from PBS control (C) by a paired t test, * P<0.01, ** P<0.001.

Fig. 5. Effects of fraction from OSCC(A) and normal tissue (B) extracts fractionated by Sephadex gel column fractionation on one-way mixed lymphocyte culture. Protein conc. of fractions is 5μg per 200μl culture. Dm; mitomycin-treated stimulator lymphocyte. Each point represents mean cpm ± SD of 3 repeat in triplicate cultures. ●; stimulator(Dm) vs responder(E), ○; stimulator(Dm) vs responder(F). Significance of differences from PBS control (C), * P<0.01, ** P<0.001.
Table 1. Responses of normal and OSCC-sensitised lymphocytes to acid dissociated sub-fractions of the fractions obtained by Sephadex G-150 column chromatography

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sub-fraction MW x 10^3 d</th>
<th>Sub-fraction (^a) alone</th>
<th>Sub-fraction</th>
<th>plus PHA-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NL</td>
<td>OSL</td>
<td></td>
</tr>
<tr>
<td>PBS Control</td>
<td>1.3 ± 0.3 (^b)</td>
<td>1.6 ± 0.3</td>
<td>83.5 ± 23.1</td>
<td>174.1 ± 44.7</td>
</tr>
<tr>
<td>Tumour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.1 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>78.1 ± 19.4</td>
<td>187.9 ± 39.4</td>
</tr>
<tr>
<td>I 10 – 100</td>
<td>1.4 ± 0.4</td>
<td>4.3 ± 0.9**</td>
<td>82.0 ± 21.8</td>
<td>190.3 ± 45.2</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.4</td>
<td>91.8 ± 26.9</td>
<td>170.4 ± 32.1</td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.0 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>75.6 ± 19.8</td>
<td>191.1 ± 41.8</td>
</tr>
<tr>
<td>II 10 – 100</td>
<td>1.4 ± 0.3</td>
<td>2.2 ± 0.5*</td>
<td>81.7 ± 23.5</td>
<td>177.5 ± 34.3</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.4</td>
<td>77.8 ± 21.8</td>
<td>184.6 ± 41.8</td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.2 ± 0.2</td>
<td>1.9 ± 0.6</td>
<td>79.9 ± 19.8</td>
<td>170.4 ± 43.6</td>
</tr>
<tr>
<td>III 10 – 100</td>
<td>1.8 ± 0.5*</td>
<td>5.8 ± 1.1**</td>
<td>88.1 ± 26.8</td>
<td>195.8 ± 49.3</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.4</td>
<td>84.6 ± 28.9</td>
<td>191.3 ± 47.6</td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>79.4 ± 20.6</td>
<td>186.4 ± 38.7</td>
</tr>
<tr>
<td>IV 10 – 100</td>
<td>1.1 ± 0.4</td>
<td>2.3 ± 0.7*</td>
<td>84.1 ± 24.1</td>
<td>179.8 ± 37.4</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>91.7 ± 30.6</td>
<td>189.7 ± 42.9</td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>75.1 ± 18.9</td>
<td>159.4 ± 32.8</td>
</tr>
<tr>
<td>V 10 – 100</td>
<td>0.8 ± 0.2**</td>
<td>1.0 ± 0.2**</td>
<td>48.5 ± 16.8**</td>
<td>89.6 ± 29.8**</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.5 ± 0.3</td>
<td>1.2 ± 0.3*</td>
<td>74.5 ± 18.4</td>
<td>131.4 ± 31.3*</td>
</tr>
<tr>
<td>Normal tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.5</td>
<td>74.9 ± 19.8</td>
<td>178.1 ± 30.9</td>
</tr>
<tr>
<td>iii 10 – 100</td>
<td>1.5 ± 0.4</td>
<td>1.6 ± 0.3</td>
<td>88.1 ± 28.3</td>
<td>180.4 ± 37.4</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>82.6 ± 24.6</td>
<td>175.6 ± 40.8</td>
</tr>
<tr>
<td>&gt;100</td>
<td>1.4 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>77.9 ± 18.7</td>
<td>191.4 ± 44.5</td>
</tr>
<tr>
<td>v 10 – 100</td>
<td>1.1 ± 0.4</td>
<td>1.6 ± 0.3</td>
<td>79.4 ± 20.4</td>
<td>185.4 ± 43.6</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1.5 ± 0.4</td>
<td>1.1 ± 0.4</td>
<td>86.4 ± 23.9</td>
<td>178.8 ± 40.9</td>
</tr>
</tbody>
</table>

NL = Normal lymphocytes, OSL = OSCC – sensitised lymphocytes

a, 40 \(\mu\)l of subfractions was added to 200 \(\mu\)l culture.

b, Numbers represent the mean cpm \(^3\)H x 10^3 ± SD of lymphocytes from 3 different sources in triplicate cultures.

Significance of differences from PBS control by a paired t test, \(* P<0.01, \,** P<0.001.\n
gel electrophoresis on lymphocyte blastogenicity

Sephadex G-150 fractions I, III and V of OSCC extracts, and fraction i, iii and v of normal tissue extracts were fractionated further by gradient gel electrophoresis. The eluates (40 \(\mu\)l/200
μl) from 14 strips were tested for blastogenesis of OSL and NL. As shown in Fig. 6, no significant changes in lymphocyte blastogenesis to both OSL and NL was observed in any of 14 eluates from normal tissue fractions. Eluates No 4 to 6 of OSCC fraction III showed very significant blastogenesis to OSL while eluates No 8 and 9 of OSCC fraction I revealed transient response. NL showed no changes with any eluates of these fractions. Eluates No 9 to 11 of OSCC fraction V showed significantly lower cpm with OSL and NL, compared with medium controls. The decreasing of cpm was more evident in OSL than NL.

![Diagram](image)

**Fig. 6.** Blastogenicity of normal (●) and OSCC-sensitised (○) lymphocytes to eluates obtained from gradient polyacrylamide gel electrophoresis of OSCC Fractions I, III and V. 40μl eluate from each slice was added to 200μl culture.

* Range of cpm of normal and OSCC-sensitised lymphocytes to eluates from normal tissue Fractions i, iii and v. Range (cpm) of controls for medium and eluting buffer to both lymphocytes = 1,638 ± 194. Each point represents mean cpm of lymphocytes from 3 different sources in triplicate cultures.


DISCUSSION

Jun et al.\(^{11}\) have reported regular enhancement and increased metastases when sheep bearing OSCC were injected with crude extracts of OSCC, and subsequent works\(^{7,8,9,10,12}\) showed that, during continued growth of OSCC, a significant cell-mediated immunity to one tumour fraction was generated, but that the expression of this immunity was suppressed by a second tumour fraction which caused immunosuppression. Similar results have also been described in many other tumours by various investigators\(^{1,2,4,6,26}\).

The aim of the work described was to determine if the extracts of OSCC contained tumour specific and immunosuppressive components. To clarify the immunological properties of the fractions from 3M KCl extracts of OSCC, such techniques as lymphocyte blastogenicity, acid dissociation-ultrafiltration and gradient polyacrylamide gel electrophoresis were employed.

The elution profile of Sephadex G-150 fraction of the OSCC extracts (Fig. 1A and B) was found very similar to the results described in the previous report\(^7\). However, it was more evident by the present studies that fraction III contains immunostimulatory proteins while fraction V is associated with immuno-suppressive materials (Fig. 2, Fig. 3, Fig. 4 and Fig. 5). By immunoelectrophoresis against rabbit anti-sheep serum, fractions I, II and III of OSCC were revealed to contain a large amount of immunoglobulins either Ig G or Ig M (Fig. 1). Koneval et al.\(^{13}\) have demonstrated immunoglobulins in tumour and marginal tissues of squamous cell carcinoma of the head and neck of human, and suggested that growth and metastasis of tumour could be related to the presence of immunoglobulins. There are more reports suggesting that immunoglobulins play an important role as a blocking factor in tumour immunity\(^{1,21,30}\). Albin et al.\(^1\) and Murray et al.\(^{21}\) have indicated that the presence of blocking factors, probably immunoglobulins or immune complexes, which inhibited cell-mediated cytotoxicity against tumour cells appear to provide one of the important mechanisms for escape of tumours from the immune cytotoxic response of the host. Conversely, some investigators\(^{24,26}\) have reported that immunisation with cell-free fractions of neoplasms has resulted in the production of tumour specific antibody inducing antibody-dependent cell cytotoxicity or complement-dependent serum cytotoxicity against tumour cells. However, the present evidences seem to be not enough to elucidate whether the immunoglobulins in the fractions relate to the enhancement and metastasis of OSCC or tumour regression following cyclophosphamide injection\(^9\).

When the fractions were acid-dissociated and separated into three groups of molecular weights as indicated in Table 1, the immunosuppressive materials were detected in the subfractions of 10,000 to 100,000 daltons and <10,000 daltons of fraction V, and immunostimulating proteins, probably tumour specific antigens, in the subfractions of 10,000 to 100,000 daltons of fraction I, II, III and IV, but mainly in the fraction III. Glasgow et al.\(^5\) have indicated that dialfiltration of fraction I from the sera of various cancer patients yielded a fraction (<10,000 daltons) that was highly immunosuppressive, and that anergy in cancer appears to be correlated with the presence of a circulating immunosuppressive serum component. In mouse tumour system, an immunosuppressive peptide fraction of the smaller molecular weights has been isolated by means of gel filtration, membrane partition and ion exchange chromatography\(^{5,22}\). Moreover, alpha-feto-proteins\(^{6,14,15,20}\) and alpha-globulin\(^3,18\) have been also pointed out as the causes for immunosuppression. Recently, some investigators\(^{16,25,27,28}\) have reported that suppressor factors specific for the methylcholanthrene-induced fibrosarcoma are closely associated with the cell mem-
brane proteins that bear the determinant encoded by I-J subregions of the murine MHC.

In accordance with the present results, it has become evident that both immunostimulatory and immunosuppressive factors in the OSCC model are connected with the cell membrane materials of OSCC, and the materials of the smaller molecular size (<10,000 daltons) cause immunosuppression in OSCC bearing sheep. In addition, OSCC-sepecific proteins could be isolated from the fraction III of Sephadex fractionation of 3M KCl extracts of OSCC. However, whether both factors inducing immunosuppression or immunostimulation in vitro does so directly, or through other pathways of induction of immunomodulation by the cooperation of T and B suppressors, or other chemicals as alphaglobulins, peptides, alpha-feto-protein and lymphocytic chalones must await further studies.

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munother. 3: 247.


