Role of Clusterin and Tumor Necrosis Factor Receptors on the Apoptosis of Prostate Cancer Cells

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**Abstract**

**Purpose:** In prostate cancer, the anti-apoptotic mechanism of sulfated glycoprotein-2 (clusterin) against tumor necrosis factor-alpha (TNF-α) receptors and the action of type 2 TNF-α receptor (TNFR2) were investigated.

**Materials and Methods:** TNF-α, agonistic-TNF type 1 receptor (TNFR1) antibody, agonistic-TNF-R2 antibody and their combination were treated in PC3 cell line with or without anti-clusterin. Cytotoxicity was assessed by trypan blue dye exclusion assay. By using flowcytometric analysis, the exact amount of apoptosis and their changes were assessed.

**Results:** Apoptosis was significantly increased in both agonistic-TNFR1 antibody and TNF-α treated cases after blocking the activity of clusterin. The more the anti-clusterin antibody added, the more the apoptosis occurred. The increase of total apoptosis was greater in TNF-α treated cells than in agonistic-TNFR1 antibody treated ones. However, there was no increase of apoptosis in agonistic-TNFR2 antibody and TNF-α with agonistic-TNFR2 antibody treated cases, respectively.

**Conclusions:** Clusterin prevents TNF-α induced apoptosis by affecting TNFR1. The difference in degree of apoptosis between agonistic-TNFR1 antibody treated cells and TNF-α treated ones suggests the possibility of the action of TNFR2. It may be associated with affinity of TNF-α to the tumor cell surface.

**Key Words:** Prostate neoplasm, Clusterin, Tumor necrosis factor-alpha, Receptors, Tumor necrosis factor, Type II

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**Introduction**

One of the representative forms of programmed cell death, apoptosis is a phenomenon that kills unnecessary cells without affecting adjacent cells for the maintenance of tissue homeostasis as well as the proliferation of cells. It is assumed that the tumor is developed or undergoes various progression stages because the abnormal suppression of apoptosis. Apoptosis is expressed by signal transduction system and the related receptors. Tumor Necrosis Factor (TNF) receptor is one of key factors that are involved in apoptosis, and it has been known as a receptor associated with the permeability of cell membrane. Tumor necrosis factor has a cytotoxic activity against various types of tumor cells. For the expression of apoptosis due to TNF-α, the complex of TNF-α and its receptor should be preceded. As the receptors which are involved in this type of binding, there are subtypes of TNF-α receptors and these include type 1 TNF receptor (TNFR1) with a molecular weight of 55 k-Da and type 2 TNF receptor (TNFR2) with a molecular weight of 75 k-Da. Of these, TNFR1 has been known to have a higher possibility that it might play a key role in the apoptosis due to TNF-α.
Clusterin is one of the glycoproteins which are synthesized in and then released from Sertoli cells and epithelial cells covering epididymis, and it has been termed as sulfated glycoprotein-2 (SGP-2), castration induced protein or testosterone repressed prostate message-2 (TRPM-2). It is a membrane stabilizing protein, which has been confirmed to transiently increased and then gradually decreased following the blockage of male hormone. It has also been reported to have a close relationship with the suppression of apoptosis.

Of various types of prostate cancer cell lines, PC3 cells were isolated from human prostate cancer cells with a bone metastasis. They release a greater amount of clusterin. Thus, they become resistant to apoptosis due to TNF-α. It has been reported, however, that PC3 cells are partly responsive to the cytotoxicity due to TNF-β. To explain an inhibitory effect of clusterin on the apoptosis, it has been hypothetized that it directly binds to TNF-α and thereby inactivates it following the formation of the complex; it competetively binds to TNF-α receptor and blocks the apoptotic pathway; and it binds to cell surface molecules such as spingomyelin and thereby blocks the signal transduction pathway due to TNF-α. On the other hand, little is known about the actions of Type 2 receptor yet. Tartaglia et al reported that Type 2 receptor interferes with the cytotoxicity due to Type 1 receptor. However, according to Espevik et al both Type 1 and 2 receptors are involved in the expression of cytotoxicity due to TNF-α. As described here, controversial opinions exist regarding the involvement of TNF-α receptors in the expression of cytotoxicity.

In the current study, by treating PC3 cells with anti-clusterin antibody and thereby suppressing their activity, with a single or combined use of TNF-α, agonistic TNFR1 antibody and agonistic TNFR2 antibody, a comparative analysis was performed for the degree of apoptosis of PC3 cells. Thus, attempts were made to demonstrate the mechanisms by which clusterin is involved in the apoptosis due to TNF-α and to elucidate the role of Type 1 and 2 TNF-α receptors.

### Materials and Methods

#### 1. The preparation and culture of cell lines

PC3 cells were purchased from The Cell Line Bank of School of Medicine of Seoul National University, and were stored in an RPMI-1640 culture medium (Sigma Chemical Co., St Louis, MO) containing a 10% fetal bovine serum (Gibco BRL, Div. of Life Technologies Inc., Grand Island, NY). Prior to the experiment, cells were aliquoted on a 6-well plate. Then, following an approximately 1-day culture in an incubator in such conditions as 37°C and 5% CO2, cells were fixed to the plate. All the drugs were administered following the synchronization of cellular differentiation by removing the fetal bovine serum one day before the initiation of experiment.

#### 2. The determination of the dose of anti-SGP-2 antibody

To rule out the interference effect of additives, such as NaN3, contained in anti-clusterin antibody (goat anti-human clusterin antibody, RDI Inc., Flanders, NJ), the dose was initiated from a concentration of 20.0 μg/ml and then gradually decreased to 1/3∼1/2 times. Following the examination, it was confirmed that there were no interference effects at a concentration of <2.5 μg/ml. According to this, the dose was used in each interval for a concentration of <2.0 μg/ml.

#### 3. The determination of the dose of TNF-α, agonistic-TNFR1 antibody and agonistic-TNFR2 antibody

In regard to the dose of TNF-α (recombinant human Tumor Necrosis Factor-Alpha, RDI Inc., Flanders, NJ), 10.0 ng/ml has been demonstrated to be the optimal one. In such a condition that the sample was treated with the anti-clusterin antibody, the dose was escalated to 2∼3 times (20.0 ng/ml and 30.0 ng/ml). According to this, at a concentration of >10.0 ng/ml, no cytotoxic effects were found to be increased following the administration of an increased dose of TNF-α. The dose of agonistic-TNFR1 antibody (goat
anti-human soluble TNF-receptor 1, RDI Inc., Flanders, NJ) was set at 10.0 μg/ml, which was based on the experimental results about human A549. An experiment was performed in the same manner as the determination of dose of TNF-α, according to which no cytotoxic effects were found to rise at a concentration of 10.0 μg/ml. The dose of agonistic-TNFR2 antibody (mouse anti-human soluble TNF-receptor 2, RDI Inc., Flanders, NJ) was set at 10.0 μg/ml, which was based on the neutral concentration of TNF-α according to manufacturer’s instructions. Besides, the dose of anti-clusterin antibody and the dose of TNF-α were set at 2.0 μg/ml and 10.0 ng/ml, respectively. Besides, after the dose of agonistic-TNFR1 antibody was increased to 10 μg/ml, 15 μg/ml, 30 μg/ml and 60 μg/ml, changes in the apoptosis were examined. According to this, no inhibitory effects on TNF-α were found to rise at a concentration of >10.0 μg/ml.

4. An analysis of cell viability through a trypan blue dye extraction assay

To monitor the changes in the number of cells and their viability depending on the treatment with each drug, more than 80% confluent cells which were fixed to a 6-well plate were treated with various concentrations of anti-clusterin antibody (0 μg/ml: control group, 1.25 μg/ml, 1.5 μg/ml, 1.75 μg/ml, 2.0 μg/ml). One hour later, they were divided into three groups and treated with a 10 ng/ml of TNF-α, a 10 μg/ml of agonistic-TNFR1 antibody and a 10 μg/ml of agonistic-TNF-α antibody, respectively. Then, samples were divided into two time categories and then cultured for 24 and 36 hours. Besides, to examine how Type 2 receptor is involved in the actions of TNF-α, samples were treated with various concentrations of anti-clusterin antibody in the same methods and one hour later, cells were treated synchronously with TNF-α 10 ng/ml and agonistic-TNFR2 antibody 10 μg/ml. Otherwise, following a concomitant treatment with agonistic-TNFR1 antibody 10 μg/ml and agonistic-TNF-2 antibody 10 μg/ml, cells were cultured for 24 hours and 36 hours. Cultured cells were rinsed with a phosphate buffer solution twice, and were suspended with a binding buffer to make a final number of cells of 1×10⁶ cells/ml. Then, a 100 μg of this solution was transferred to a 5 ml culture tube. Then, a 5 μg of annexin V-fluorescein isothiocyanate (FITC) (PharMingen Co., San Diego, CA) and a 2 μl of propidium iodide (PI) solution (PharMingen Co., San Diego, CA) were mixed in a tube and the reaction was performed in a dark room accordingly. Thereafter, following the addition of a 400 μl of binding buffer, the number of cells was measured using a Becton Dickinson FACScan™ (Lysis II Ver. 1.0) until it exceeded 10,000. Based on the results of interpretation of a FACScan™ flowcytometry, depending on the expression of Annexin V-FITC and PI, changes in the apoptosis seen following the treatment with each drug were monitored and then analyzed.

It was a principle that all the experimental procedures were repeated three times. Experimental results

% Cellular toxicity=(total cells−viable cells)/total cells.

5. An analysis of the degree of apoptosis of cells based on a flowcytometry

To analyze the degree of apoptosis of TNF-α and agonistic-TNFR1 antibody depending on the alterations in the activity of clusterin and to clarify whether TNF2 is solely involved in the apoptosis, cells were treated with anti-clusterin antibody (0 μg/ml: control group, 1.25 μg/ml, 1.5 μg/ml, 1.75 μg/ml, 2.0 μg/ml) and one hour later, they were also treated with a 10 ng/ml of TNF-α, a 10 μg/ml of agonistic-TNFR1 antibody, or a 10 μg/ml of agonistic-TNF2 antibody, respectively. Then, samples were divided into two categories and then cultured for 24 and 36 hours. Besides, to analyze the changes in the number of cells and their viability depending on the treatment with each drug, more than 80% confluent cells which were fixed to a 6-well plate were treated with various concentrations of anti-clusterin antibody (0 μg/ml: control group, 1.25 μg/ml, 1.5 μg/ml, 1.75 μg/ml, 2.0 μg/ml). One hour later, they were divided into three groups and treated with a 10 ng/ml of TNF-α, a 10 μg/ml of agonistic-TNFR1 antibody and a 10 μg/ml of agonistic-TNF-α antibody, respectively. Then, samples were divided into two time categories and then cultured for 24 and 36 hours. Besides, to examine how Type 2 receptor is involved in the actions of TNF-α, samples were treated with various concentrations of anti-clusterin antibody in the same methods and one hour later, cells were treated synchronously with TNF-α 10 ng/ml and agonistic-TNF2 antibody 10 μg/ml. Otherwise, following a concomitant treatment with agonistic-TNFR1 antibody 10 μg/ml and agonistic-TNF-2 antibody 10 μg/ml, cells were cultured for 24 hours and 36 hours. Cultured cells were rinsed with a phosphate buffer solution twice, and were suspended with a binding buffer to make a final number of cells of 1×10⁶ cells/ml. Then, a 100 μg of this solution was transferred to a 5 ml culture tube. Then, a 5 μg of annexin V-fluorescein isothiocyanate (FITC) (PharMingen Co., San Diego, CA) and a 2 μl of propidium iodide (PI) solution (PharMingen Co., San Diego, CA) were mixed in a tube and the reaction was performed in a dark room accordingly. Thereafter, following the addition of a 400 μl of binding buffer, the number of cells was measured using a Becton Dickinson FACScan™ (Lysis II Ver. 1.0) until it exceeded 10,000. Based on the results of interpretation of a FACScan™ flowcytometry, depending on the expression of Annexin V-FITC and PI, changes in the apoptosis seen following the treatment with each drug were monitored and then analyzed.

It was a principle that all the experimental procedures were repeated three times. Experimental results
were expressed as mean±SD (SD: standard deviation). Besides, statistical analysis was performed to identify the difference in the degree of apoptosis, for which Student t-test and Mann-Whitney rank sum test were used.

**Results**

PC3 cells grew in a monolayer cluster, where approximately 6~7 days elapsed for a subculture.

1. Changes in the number of cells and cell viability depending on the inhibition of clusterin activity following the treatments with TNF-α, agonistic-TNFR1 antibody and agonistic-TNFR1 antibody

   In samples which were treated with TNF-α, after 24 hours, the number of cells was significantly decreased as the concentration of anti-clusterin antibody was increased. After 36 hours, it was decreased with no respect to the use of anti-clusterin antibody in all the cases. Accordingly, there was no significant difference in this series (Fig. 1A). After 24 hours, the cell viability was decreased as the concentration of anti-clusterin antibody was increased. However, there were no significant differences compared to control group. After 36 hours, the cell viability was significantly decreased as the concentration of anti-clusterin antibody was increased (p<0.05). At a concentration of 2.0 μg/ml, nearly all the cells were ruptured (Fig. 1B).

   In samples which were treated with agonistic-TNFR1 antibody, after 24 hours, the number of cells showed no significant difference depending on the addition of anti-clusterin antibody. After 36 hours, the number of cells was decreased as the concentration of anti-clusterin antibody was increased. But this was not significantly different from the control group (Fig. 2A). After 24 hours, with no respect to the presence of anti-clusterin antibody, there was no significant difference in the cell viability. After 36 hours, however, the cell viability was significantly decreased as the use of anti-clusterin antibody was increased (p<0.05). The results were similar to samples which were treated with TNF-α (Fig. 2B).

   In samples which were treated with agonistic-TNFR2 antibody, after 24 or 36 hours, with no respect to the dose of anti-clusterin antibody, there were no significant differences in the number of cells and cell viability as compared with the control group.

2. The measurement of the early- and late-stage degree of apoptosis on FACScan

   After 24 hours following the treatment with TNF-α, as the dose of anti-clusterin antibody was increased, the end-stage and overall degree of apoptosis were sig-
significantly increased (cells undergoing the early-stage apoptosis + those undergoing the late-stage one) (p < 0.05). After 36 hours, the number of cells undergoing the early-stage apoptosis was markedly decreased. But the overall degree of apoptosis was significantly increased in all the samples which were treated with anti-clusterin antibody (Fig. 3A).

After 24 hours following the treatment with agonistic-TNFR1 antibody, in the group where a lower concentration of anti-clusterin antibody was used, the degree of apoptosis was not significantly different from the control group. In the group where a 2.0 μg/ml of anti-clusterin antibody was used, however, both the early-stage and the overall degree of apoptosis showed a significant increase as compared with the control group (p<0.05). After 36 hours, as the dose of anti-clusterin antibody was increased, cells undergoing the early-stage apoptosis showed a gradual decrease. Besides, the overall degree of apoptosis showed a significant increase in all the concentration intervals (Fig. 3B). In samples which were treated with same concentrations of anti-clusterin antibody, the degree of apoptosis after 24 hours was significantly higher following the treatment with TNF-α as compared with that with agonistic-TNFR1 antibody. After 36 hours, however, there was no significant difference.

Fig. 2. Assessment of the response of PC3 cell to the treatment of agonistic-TNFR1 antibody with varying amount of anti-clusterin antibody. (A) Cell number was counted with Coulter hemocytometer. (B) Cell viability was evaluated by trypan-blue dye extraction assay. Each column represents mean value (bars, SD) of triple replicates experiments. Anti-SGP-2: anti-clusterin antibody. *p<0.05.

Fig. 3. Effects of anti-clusterin antibody to early and total apoptosis. (A) Cases of TNF-α treatment. (B) Cases of agonistic-TNFR1 antibody treatment. Anti-SGP-2: anti-clusterin antibody. *p<0.05.
In samples which were treated with agonistic-TNFR2 antibody, after 24 and 36 hours, with no respect to the dose of anti-clusterin antibody, the early-stage and overall degree of apoptosis showed no significant difference as compared with the control group (Table 1).

In samples which were treated with synchronously with TNF-α and agonistic-TNFR2 antibody, after 24 hours, with no respect to the dose of anti-clusterin antibody, the early-stage and overall degree of apoptosis showed no significant difference as compared with the control group. In all the samples which were treated with anti-clusterin antibody after 36 hours, however, the number of cells undergoing early-stage apoptosis was decreased. The overall degree of apoptosis was significantly increased in all the intervals of concentrations of anti-clusterin antibody (Fig. 4A, 5A, 5C). After 24 hours following the treatment with same concentrations of anti-clusterin antibody, the degree of apoptosis was markedly decreased in samples which were treated synchronously with TNF-α and agonistic-TNFR2 antibody as compared with those which were solely treated with TNF-α (p<0.05). After 36 hours, however, there was no significant difference in this series (Fig. 3A, 4A).

In samples which were treated synchronously with agonistic-TNFR1 antibody and agonistic-TNFR2 antibody, the early-stage and overall degree of apoptosis were significantly increased in those treated with a 2.0 μg/ml of anti-clusterin antibody after 24 hours as compared with the control group (p<0.05). After 36 hours, as the dose of anti-clusterin antibody was increased, cells undergoing early-stage apoptosis were gradually decreased. The overall degree of apoptosis was significantly increased in all the concentration intervals (Fig. 4B, 5B, 5D). After 24 and 36 hours following the treatment with same concentrations of anti-clusterin antibody, the degree of apoptosis showed no significant difference between samples which were

Table 1. Result of flowcytometric analysis of apoptosis of PC3 cells after treatment of agonistic-TNFR2 antibody with varying amount of anti-clusterin antibody. Data are expressed as a mean with SD

<table>
<thead>
<tr>
<th>Treatment duration</th>
<th>Anti-clusterin concentration (μg/ml)</th>
<th>FACScan</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Early apoptosis</td>
<td>Total apoptosis</td>
</tr>
<tr>
<td>24 hours</td>
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<td>9.2±3.6</td>
<td>15.7±1.2</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>1.75</td>
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<td>19.4±3.5</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>12.0±2.2</td>
<td>21.0±2.9</td>
</tr>
<tr>
<td>36 hours</td>
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</tr>
<tr>
<td></td>
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<td>2.00</td>
<td>23.9±2.0</td>
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Asterisks (*) indicate significant increase compared with control group (p<0.05). Anti-clusterin: anti-clusterin antibody.

Fig. 4. Effects of anti-clusterin antibody to early and total apoptosis. (A) Cases of TNF-α with agonistic-TNFR2 antibody treatment. (B) Cases of agonistic-TNFR1 antibody with agonistic-TNFR2 antibody treatment. Anti-SGP-2: anti-clusterin antibody. *p<0.05.
solely treated with agonistic-TNFR1 antibody and those which were treated synchronously with agonistic-TNFR1 antibody and agonistic-TNFR2 antibody.

**Discussion**

Most of the cells forming the prostate cancer are dependent on androgen, and these cells undergo apoptosis when the secretion of androgen was blocked. That is, androgen has an inhibitory effect on apoptosis. When they are decreased below a certain level, one of the representative forms of programed cell death, apoptosis occurs.\(^{12,13}\) As the representative death receptors causing a programed cell death, there are such receptors as Fas/Apo-I, TNF receptor and transforming growth factor-\(\beta\) receptor. PC3 cells which were used in the current study are cell lines which were isolated from prostate cancer with bone metastasis, and they are less vulnerable to the alterations in androgen. As compared with LNCaP cells which were isolated from prostate cancer with metastasis to supraclavicular lymph node, PC3 cells release a greater amount of clusterin. This has been known to block the cytotoxicity due to TNF-\(\alpha\) in prostate cancer cells.\(^{14}\)

Clusterin is one of the membrane stabilizing glycoproteins, and it is a dimer with a molecular weight of approximately 75-kDa consisting of two subunits (\(\alpha\) and \(\beta\)).\(^{15-17}\) There are two clusterin isoforms in human. A nuclear form of clusterin (nCLU) is pro-apoptotic, and a secretory form (sCLU) is pro-survival.\(^6\) Clusterin suppresses autophagic lysis due to epithelial cells during apoptosis. Tumor progression is associated with a loss of apoptotic potential which is related to the expression of clusterin.\(^7,18\) Following the castration of normal rats, the concentration of clusterin mRNA was increased in the prostate gland.\(^{19}\) An increased level of clusterin has been reported to have a relationship with apoptosis in the prostate gland following the castration of normal rats.\(^{20,21}\) Since then, experiments...
have been performed induce the apoptosis using TNF-α in cultured prostate cancer cells. Thus, it has been confirmed that the concentration of clusterin is elevated prior to the emergence of DNA fragmentation as one of the typical findings of apoptosis and it is decreased as the time elapses following the emergence of DNA fragmentation.5,8

The cell viability is decreased by the action of TNF-α or agonistic-TNFR1 antibody as the concentration of anti-clusterin antibody is increased. In the current study, the cell viability was significantly decreased after 36 hours. However, the cell viability was not so decreased after 24 hours. This finding suggests that late-stage apoptosis rather than early-stage apoptosis acts on the cells much more.

Tumor necrosis factor showing a cytotoxic effect during apoptosis is synthesized by the activated macrophages and lymphocytes. TNF-α and -β are also termed as cachexin and lymphotoxin, respectively. Of these, cell membrane receptors which are involved in binding to TNF-α are polypeptides occupying the single membrane-spanning region which is composed of approximately 145 amino acids. By showing the similarity of cystein-rich extracellular portion with such molecules as TNF-β, FAS, nerve growth factor and B cell activation protein, they are classified as a cytokine receptor gene family.18,22-24 Besides, activated TNF-α receptors are also known to induce the apoptosis via a sphingomyelin pathway in the same manner as these substances.2,25 The most important step in the expression of cytotoxicity due to TNF-α is an oligomerization where the extracellular regions of receptors form polymers through a cross-linking after TNF-α binds to its receptor.3 It is well established that the cytotoxicity of TNF-α originates from its binding to Type I receptor. Following the formation of complex between TNF-α and Type I receptor, FAS associated death domain (FADD) binds to TNFR1 death domain (TNFR1-DD) and this eventually activates the process of apoptosis.26 There are also some reports that apoptosis was induced with the use of antibodies which selectively activate Type 1 TNF-α receptor.27 Also in the current study, following the blockage of clusterin in PC3 cells and the subsequent administration of antibodies which selectively activate Type 1 TNF-α receptor, the apoptosis was observed to occur. This confirms that the activation of Type 1 TNF-α receptor plays a major role in the expression of cytotoxicity due to TNF-α. It has been reported that Type 2 receptor has various functions such as the promotion of the proliferations of thymocytes.28 However, the role of Type 2 receptor in the expression of cytotoxicity due to TNF-α has been mostly unknown. According to an experiment that selectively blocks TNF-α receptors with the use of monoclonal antibodies against Type 1 or 2 receptors, it was reported that both Type 1 and 2 receptors are involved in the expression of cytotoxicity due to TNF-α;11 the activation of Type 2 receptors increased the affinity of TNF-α to Type 1 receptors; and this eventually leads to the increased expression of cytotoxicity.11,29 Another study also reported that the activation of Type 2 receptor reduced the amount of TNF-α which is needed for the expression of cytotoxicity, according to which this was because the concentration of TNF-α is elevated on the surface, following the prompt binding between Type 2 receptor and TNF-α and sequestration of them, and this eventually promoted binding between Type 1 receptor and TNF-α.30 Also in the current study, in the group where agonistic-TNFR2 antibody was solely administered following the blockage of clusterin, there were no changes in the degree of apoptosis. This confirmed that Type 2 TNF-α receptor had no direct impact on the cytotoxicity. Besides, in samples which were treated synchronously with agonistic-TNFR1 antibody and agonistic-TNFR2 antibody following the blockage of clusterin, there were almost no apoptotic events. Based on these findings, it can be inferred that agonistic-TNFR2 antibody binds to the receptor and thereby interferes with the formation of complex between TNF-α and its receptor. To put this in another way, it is presumed that Type 2 receptor raises the affinity of TNF-α and thereby promotes a prompt binding to Type 1 receptor.

A flowcytometry is based on the characteristics of
Annexin V and PI. Phosphatidylserine is one of the constituents of cell membrane, and it has a polarity to the internal side of cell membrane in normal cells. It is exposed to the external side of cell membrane prior to DNA fragmentation in the early stage of apoptosis. A substance which irreversibly binds to phosphatidylserine, Annexin V can confirm the apoptosis at earlier times as compared with the previous methods. PI can confirm the disruption of cell membrane, a phenomenon occurring in the late-stage of apoptosis. Its synchronous use with Annexin V can therefore be used to differentiate between apoptosis and necrosis, which is also effective in differentiating between the early-stage apoptosis and the late-stage one. Also in the current study, the presence of apoptosis induced by TNF-α could be measured at earlier times with the use of a flowcytometry. By differentially measuring the early and late-stage apoptosis, an analysis could efficiently be performed to assess changing trends in the time-dependent apoptosis.

**Conclusions**

Through the current study, the inhibitory effect of clusterin on the apoptosis due to TNF-α in PC3 cells was confirmed to originate from TNFR1. As samples which were treated with TNF-α, in those which were treated synchronously with TNF-α and agonistic-TNFR2 antibody, the degree of apoptosis was markedly decreased. There was a statistical significance in this series. To put this in another way, it can be inferred that agonistic-TNFR2 antibody binds to TNFR2 and then interferes with the formation of complex between TNF-α and the receptors and this eventually leads to the inhibition of apoptosis. These findings indicate that TNFR2 raises the affinity of TNF-α on the surface of tumor cells and thereby promotes the formation of complex between TNF-α and the receptors. Further studies are warranted to additionally examine whether clusterin would have an effect on TNF-α or TGF-β receptors in LNCaP cell lines. Based on the results of current study, this would be useful in predicting the prognosis of prostate cancer based on an understanding of the development and suppression of it as well as treating it accordingly.

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**REFERENCES**

20) Nickerson T, Pollak M, Huynh H. Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2,3,4 and 5. Endocrinology 1998;139:807-10
30) Tartaglia LA, Pennica D, Goeddel DV. Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem 1993;268:18542-8