Production of Human Antithrombin-III in a Serum-free Culture of CHO Cells

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A simple method was developed to establish serum-independent Chinese hamster ovary (CHO) cells that grew and secreted high levels of human antithrombin-III (AT-III). First, human AT-III and mouse dihydrofolate reductase (DHFR) cDNAs were transfected into DHFR-deficient CHO cells. Transfected cells were treated with increasing concentrations of methotrexate (MTX) and clones secreting high levels of AT-III (10—20 μg/ml/3 day) in a serum-containing medium were obtained. Serum-independent clones were derived from the serum-dependent clones by simply culturing the cells for a few weeks in a serum-free medium. In a serum-free medium the established serum-independent clones grew at normal rate and produced almost equivalent amount of AT-III to that of the serum-dependent, parent clones. In addition, AT-III from the serum-independent clones has specific activity similar to that of plasma-derived AT-III.

AT-III is a protease inhibitor which inhibits blood coagulation factors such as thrombin, IXa, Xa, XIa, and XIIa. Human plasma contains a large quantity of AT-III and plasma-derived AT-III has been widely used for the treatment of DIC (disseminated intravascular coagulation) and hereditary AT-III deficiency. 5-9 Production or recombinant human AT-III has already been reported by several groups.10-16 Prokaryotic (Escherichia coli) or eukaryotic (yeast or mammalian) expression systems were used, but only the AT-III produced by mammalian cells was as active as plasma-derived AT-III in the presence of heparin.13-16 This implies that post-translational modifications of AT-III, such as protein folding and attachment of sugar chains, are pivotal for the activity of human AT-III.

For the commercial-scale production of human AT-III by mammalian cell culture, high productivity and low manufacturing cost are required because AT-III is abundant in human plasma and easily obtained. The presence of serum in the culture medium, however, raises the manufacturing cost and creates additional difficulty in the product purification steps. Serum-free culture of the CHO cells that produced AT-III has already been demonstrated,14 but troublesome procedures were required, in which the cells were alternatively cultured in a serum-containing and a serum-free media at 2-day intervals. We describe here a simple method for the establishment of serum-independent CHO clones which grow and produce human AT-III in a serum-free medium. AT-III productivity of the serum-independent clones was almost equivalent to that of the serum-dependent, parent clones. Besides, AT-III derived from the serum-independent clones had specific activity similar to that of the plasma-derived AT-III.

Materials and Methods

Cloning of human AT-III cDNA and construction of the expression plasmid. Human AT-III cDNA was isolated from a human liver cDNA library using synthetic DNA probes with published sequences.10 Nucleotide sequence of the cDNA agreed with the published sequence,10 except for 3 base replacements that didn’t change the deduced amino acid sequence.

AT-III expression plasmid in mammalian cells, pTY007 (Fig. 1), was constructed as briefly described below. First, mouse DHFR cDNA of the plasmid pSV2dhfr17 was replaced with human AT-III cDNA by several steps. The resultant plasmid (pTY006) contained the early promoter of simian virus 40 (SV40), human AT-III cDNA, and the polyadenylation signal of SV40. Secondly, the plasmid pSV2dhfr was modified by replacing the SV40 early promoter with a 0.8-kilobase (kb) human urokinase promoter18 to construct plasmid pTY006. From the plasmid pTY006, a 2.9-kb fragment composed of the human urokinase promoter, mouse DHFR cDNA, and the polyadenylation signal of SV40 was isolated. The fragment was inserted into the 3′-end of the SV40 polyadenylation signal of pTY006 to construct the plasmid pTY007.

Expression and amplification of AT-III production in CHO cells. Plasmid pTY007 was transfected into mutant CHO cells which lacked DHFR activity and required glycine, a purine base, and thymidine for growth by the calcium phosphate method with a glycerol treatment.19 Transfected cells were cultured for 72 hr in alpha medium (Gibco) containing 10% fetal calf serum (FCS), ribonucleosides, and deoxribonucleosides. Then the cells were split 1:6 in 10-cm petri dishes and cultured with a selective medium made from alpha medium with 10% FCS but lacking nucleosides. After a few weeks of cultivation DHFR-positive colonies appeared.21 Individual colonies were picked up, seeded into 96-well plates, and cultured until the cells became confluent. Culture media of the confluent cells were assayed for AT-III content by enzyme immunoassay (EIA) to select AT-III secreting clones.

The AT-III secreting clones were seeded into 6-well plates at 1 x 10⁴ cells per well with the selective medium containing 5 mM of MTX. When the cells became confluent, 1 x 10⁴ of the cells were recovered and reseeded into the selective medium containing 2-4 fold higher concentrations of MTX. The procedures were repeated several times until most of the clones acquired 2 to 5 μM-MTX resistance.

Establishment of AT-III producing cells which grow in serum-free culture. AT-III secreting cells were seeded into 96-well plates at 100 cells per well with a serum-free medium (GCMM01), which was made from RPMI-1640 medium (Nissui) containing 0.5% Bactepotein (Difco), 0.1% human serum albumin, 1 mg/liter of insulin, 10 mg/liter of transferrin, 13 mg/liter of hypoxanthine, 4 mg/liter of thymidine, 35 mg/liter of proline, 4 μg/liter of sodium selenite, and 130 μg/liter of vitamin E. The cells were cultured for a few weeks with medium changes at 2-3-day intervals until the cell number reached 10⁵/ml. Then the cells were harvested and cultured in a larger flask.

Assay for human AT-III. In the assays for human AT-III, purified AT-III from human plasma (Green Cross) was used as a standard. Anti-thrombin activity of AT-III in the presence of heparin (heparin cofactor activity)
was measured by a commercial kit using bovine thrombin and a chromogenic substrate (International Reagent). Measurement of AT-III was done by a solid-phase ELISA using rabbit anti-AT-III antibody (Dakopatts) and peroxidase-conjugated rabbit anti-AT-III antibody (Dakopatts). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli and the gel was silver stained. Western blotting was done by a general method. Deglycosylated AT-III was obtained by incubation of 100 ng of purified AT-III with 2.5 units of glycopeptidase F (Takara Shuzo) at 37°C for 16 hr in a reaction buffer composed of 0.1 M phosphate, pH 6.1, 0.05 M Na₂EDTA, 1% Nonidet P40, and 0.1% SDS.

**Purification of human AT-III**. Human AT-III was affinity-purified by the method of Molloy with a little modification. For the purification of AT-III produced by a serum-independent clone, 200 to 240 ml of the serum-free culture medium was concentrated to 20 ml by ultrafiltration. The concentrate was mixed with an equal volume of 2 x column buffer (1 M NaCl, 1% Tween 80, 0.1 M Tris-HCl, pH 7.5) and put on an affinity column (1.4 x 2.6 cm) that was packed with CNBr-activated Sepharose 4B (Pharmacia) coupled with anti-human AT-III antibody (Dakopatts). The column was washed with 10 volumes of 1 x column buffer (0.5 M NaCl, 0.5% Tween 80, 0.05 M Tris-HCl, pH 7.5) and AT-III was eluted with 4.5 M MgCl₂, 0.5 M Tris. The eluates were immediately diluted with 2 volumes of water and ultrafiltered. Thus purified AT-III was stored in 10 mM ammonium acetate solution at 5°C.

For the purification of AT-III produced by a serum-dependent clone, the culture medium was first mixed with 3 volumes of saturated ammonium sulfate solution to precipitate AT-III. The precipitate was recovered by centrifugation, dissolved into a small volume of 10 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer. The dialysate was mixed with an equal volume of the column buffer and put on the affinity column. AT-III was purified on the column as described above.

**Results**

**Expression of human AT-III cDNA in CHO cells**

Human AT-III cDNA was isolated from a human liver cDNA library, cloned into a mammalian expression plasmid (pTY007, Fig. 1) and expressed in DHFR-deficient CHO cells. Plasmid pTY007 carried two expression units for human AT-III and mouse DHFR, which were expressed under the control of the SV40 early promoter and human urokinase promoter, respectively. Since the urokinase promoter seemed to be a fairly weak promoter in CHO cells, it was expected that the dhfr cDNA was highly amplified by MTX treatment. The flanking AT-III cDNA would be highly coamplified accordingly, which would result in high expression level of AT-III.

DHFR-deficient CHO cells were transfected with pTY007 and 220 independent, DHFR-positive clones were isolated. About 70% of the clones secreted AT-III into the medium at a rate of 10 to 200 ng/10⁶ cells/day. To enhance the production level of AT-III, 40 clones with high productivity were chosen from the 220 clones and were treated with increasing concentrations of MTX. Starting from 5 nM, MTX concentrations were raised to 20, 50, 150, 400, 1000, 2000, and 5000 nM. AT-III productivity of the clones increased correspondingly and those of the highest clones with 2 to 5 μM MTX resistance reached 2000 to 4000 ng/10⁶ cells/day, which suggested that the productivity was increased more than 100-fold by the MTX treatment.

**Establishment of serum-independent CHO cells producing human AT-III**

Five clones that produced high levels of AT-III in a serum-containing medium were seeded into 96-well plates at 100 cells/well with a serum-free medium (GCM001). Cells grew in some wells and the concentration reached 10²⁶ ml after 3 to 4 weeks of cultivation. The number of the wells in which cells grew varied depending on the clones, from 9 to 84% of the seeded wells (Table I). This suggested that at least 0.1% of the seeded cells became serum-independent, because each well was seeded with 100 cells. Most of the established serum-independent subclones produced rela-

**Table I. Derivation of Serum-independent Clones from Serum-dependent, Parent Clones**

<table>
<thead>
<tr>
<th>Clone number</th>
<th>MTX-resistance (μM)</th>
<th>Seeded (cells growing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>1</td>
<td>192 (161, 83.9%)</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>192 (85, 44.3%)</td>
</tr>
<tr>
<td>66</td>
<td>1</td>
<td>192 (56, 29.2%)</td>
</tr>
<tr>
<td>55</td>
<td>5</td>
<td>192 (18, 9.4%)</td>
</tr>
<tr>
<td>61</td>
<td>5</td>
<td>192 (50, 26.0%)</td>
</tr>
</tbody>
</table>

- AT-III productivity of the clones in 10% FCS-containing medium was about 2,000 ng/10⁶ cells/day.
- Number of wells in which serum-dependent cells were seeded into 96-well plates at 100 cells/well.
- Number of wells in which serum-independent cells were growing after 2 to 4 weeks of cultivation in GCM001.

**Table II. AT-III Productivity of Serum-dependent, Parent Clones and Serum-independent Subclones**

<table>
<thead>
<tr>
<th>Serum-dependent parent clone</th>
<th>Serum-independent subclone</th>
<th>AT-III productivity (μg/ml/3 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#18</td>
<td>#18-13</td>
<td>11.0</td>
</tr>
<tr>
<td>#18</td>
<td>#18-48</td>
<td>19.0</td>
</tr>
<tr>
<td>#31</td>
<td>#31-13</td>
<td>8.6</td>
</tr>
<tr>
<td>#31</td>
<td>#31-26</td>
<td>13.0</td>
</tr>
</tbody>
</table>

- Cells were seeded at 10²⁶ cells/ml in 6-well plates and cultured for 3 days. AT-III contents of the culture media were measured.
- Serum-independent subclones derived from clone #18.
- Serum-independent subclones derived from clone #31.

**Fig. 1.** Structure of the Expression Plasmid pTY007.

Construction of the plasmid is shown in Materials and Methods. Abbreviations: Puy, early promoter of SV40; poly(A), SV40-derived polyadenylation signal fused with splicing junction; Pux, human urokinase promoter.
Fig. 2. Morphological Appearance of Two CHO Clones in Culture.
Serum-independent clone (M31-7) (A) and its serum-dependent, parent clone (M31) (B) were seeded at 10^5 cells/ml and cultured for 2 days in a serum-free medium (GCM001) and 10% FCS-containing medium, respectively. Original magnification 100 x.

Fig. 3. SDS-PAGE and Western Blotting Analyses of AT-III.
Purified AT-III derived from human plasma (lanes 1, 3, 5, and 8), serum-independent clone M31-7 (lanes 2 and 4), and the two serum-dependent clones M11 (lanes 6 and 9) and M31 (lanes 7 and 10) were analyzed by SDS-PAGE (A) and Western blotting (B) before (lanes 1, 2, 5, 6, and 7) or after (lanes 3, 4, 8, 9, and 10) deglycosylation. A hundred ng each of the purified human AT-III and molecular mass standards (M) was electrophoresed. Lower band of 35 kDa which co-migrated with deglycosylated AT-III (lanes 3, 4, 8, 9, and 10 of A)) corresponds to glycopeptidase F. Molecular mass markers in kDa are shown on the left.

…tively low level of AT-III (<1 μg/ml/3 day). AT-III productivity of the several subclones, however, was equal to or a little better than those of their serum-dependent, parent clones (Table II).

Some of the serum-independent subclones grew in suspended conditions and clumped in GCM001, and the others grew adherent to the plate (Fig. 2). Doubling times of the clones were around 1.5 days. When serum-independent subclones in Table II were reseeded into 96-well plates at 1 cell/well with GCM001, cells grew in most of the wells.

Characterization of AT-III produced by serum-independent CHO cells
AT-III was purified to homogeneity by affinity chromatography24 from the culture media of a serum-independent subclone (M31-7) and its serum-dependent, parent clone (M31). Purification steps for the serum-free culture medium of clone M31-7 are summarized in Table III. Yield of the eluted AT-III from the affinity column was high (50.7%), but the yield greatly decreased to 16.4% after ultrafiltration, presumably because of adsorption of AT-III to the membrane. As a result 146 μg of the purified AT-III was obtained starting from 200 ml of the serum-free culture.

### Table III. Purification of AT-III from the Culture Medium of a Serum-independent Subclone

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>AT-III activity (units)</th>
<th>Yield (%)</th>
<th>Specific activity (units/mg)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium*</td>
<td>200</td>
<td>867</td>
<td>4.81</td>
<td>100</td>
<td>0.0055</td>
<td>1</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>20</td>
<td>NT</td>
<td>4.84</td>
<td>101</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Affinity column</td>
<td>50</td>
<td>NT</td>
<td>2.44</td>
<td>50.7</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>1.25</td>
<td>0.146</td>
<td>0.79</td>
<td>16.4</td>
<td>5.41</td>
<td>984</td>
</tr>
</tbody>
</table>

* Medium of subclone M31-7 cultured for 3 days in GCM001.
* Not tested.
medium. In contrast to the serum-free medium, recovery of the AT-III from the 10% FCS-containing culture medium of clone #31 was difficult and the yield of the eluted AT-III from the affinity column was 22%. Specific activity of the purified AT-III from subclone #31-7 was 5.41 units/mg protein, in good agreement with the values of AT-III from the parent clone #31 (5.41 units/mg protein) and from human plasma (5.88 units/mg protein).

Figure 3(A) shows the results of SDS-PAGE of the purified AT-III before and after deglycosylation. Purified AT-III from a serum-independent subclone (#31-7) gave a diffused band of 58—62 kilodaltons (kDa) with a dense region at 60—62 kDa. In contrast, AT-III from serum-dependent clones showed two bands of 60 and 62 kDa. Plasma-derived AT-III gave the sharpest band of 61 kDa. After deglycosylation all the purified AT-IIIIs gave a very sharp band of 50 kDa which was in close agreement with the expected value from the amino acid sequence. The same results were obtained in Western blotting analysis (Fig. 3(B)), suggesting that AT-IIIIs derived from the serum-independent clone, serum-independent subclones, and human plasma had similar polypeptide chains and heterogeneous sugar moieties.

Discussion

In this paper we described a novel method for the establishment of CHO cells that grew and produced human AT-III in a serum-free medium. Zettlmeissl et al. has already reported the production of human AT-III by CHO cells in a serum-free medium. In that case, however, cells didn't grow in a serum-free medium and required recovery culture in a serum-containing medium. In contrast, CHO cells established by this method grew in a serum-free medium and thereby didn't require serum for the growth and the production of AT-III, which decreased the cost and the period for manufacturing AT-III.

Serum-independent clones are usually established by stepwise adaptation of serum-dependent clones to a medium containing lower concentrations of serum, and finally to a serum-free medium. The adaptation, however, usually requires several months or more and troublesome procedures. In contrast to the conventional method, this method for the establishment of serum-independent CHO cells is simpler and more convenient. Serum-independent cells can be easily established within a month by culturing serum-dependent cells in a serum-free medium (GCM001). Although serum-independent Namalwa cells that produced recombinant erythropoietin could be established by a similar method, we would like to emphasize that this method was used for the establishment of serum-independent DHFR-deficient CHO cells and their transformed cells. As DHFR-deficient CHO cells are the host that is most widely used for the production of recombinant proteins, the establishment of the serum-independent clone is valuable. This method is reproducible, because we have successfully established other kinds of serum-independent CHO clones by it, which produced, for instance, modified urokinase-type plasminogen activators.

Another point of this method is that the AT-III productivity of some serum-independent clones is equal to or even better than that of the serum-independent, parent clone (Table II) and the product is biologically active. In addition, some clones established by this method grew in suspended conditions (Fig. 2). It was also reported that wild-type CHO cells, as well as HeLa cells (human cervical carcinoma) and KB cells (human bronchial carcinoma), grew as stationary suspensions in a medium composed of alpha medium, Bactopeptide, and insulin. Suspension culture has many advantages over monolayer culture. In suspension culture it is very easy to change the medium and to achieve high cell density artificially, and thereby it is thought to be suitable for large-scale culture. We are now examining the possibility of the long-term suspension culture of the established serum-independent cells.

Specific activity of the AT-III produced by a serum-independent clone was in close agreement with the values of the AT-III from a serum-dependent clone and human plasma (Table III). In addition, AT-III from the serum-independent clone formed a stable thrombin-AI-III (TAT) complex with human thrombin (data not shown). SDS-PAGE and Western blotting analyses showed that purified AT-III from the serum-independent clone gave a diffused band of 58 to 62 kDa, but the main portion co-migrated with plasma-derived AT-III (Fig. 3). Zettlmeissl et al. also reported that the recombinant AT-III derived from a serum-dependent CHO cell gave a diffused band in SDS-PAGE with an average molecular mass of 60 kDa. These results suggest that AT-III derived from the serum-independent clones is similar to plasma-derived AT-III.

Broad distribution of the molecular mass of AT-III derived from the serum-independent clones must be attributed to the heterogeneity of sugar chains attached to the AT-III. This was concluded by SDS-PAGE and Western blotting analyses in which deglycosylated AT-III from serum-independent clones, as well as deglycosylated AT-III from serum-dependent clones and human plasma, gave a sharp band of 50 kDa (Fig. 3). In addition, it is noted that the molecular mass distribution of AT-III from the serum-independent clone was somewhat different from that of the serum-dependent, parent clone. This suggests that the sugar chain attached to AT-III had changed during the processes in which serum-dependent cells became serum-independent, or in which serum-independent cells were cultured for a long time in a serum-free medium. The latter possibility may be important, because long term cultivation of CHO cells, which produced human interferon-γ, in a serum-free medium increased the proportion of partially and non-glycosylated interferon-γ with a concomitant decrease of fully glycosylated products. Heterogeneity of the sugar chains didn't affect the biological activity of AT-III, however, because all the AT-IIIIs had nearly the same specific activities.

References