Determination of thymidine incorporation activity in the sera from patients with short stature

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Abstract. Serum activity that stimulates DNA synthesis was determined in 130 patients of short stature, 6 with acromegaly, and 5 with anorexia nervosa, using a technique to measure [\textsuperscript{3}H]thymidine incorporation into cultured human fibroblasts. The stimulation of thymidine incorporation into the fibroblasts, abbreviated as thymidine incorporation activity (TIA), was significantly lower than normal (\(P < 0.001\)) in 18 untreated children with GH-deficiency (mean \(0.63 \pm 0.14\) U/ml, mean \(\pm SD\)), and in 5 patients with anorexia nervosa (mean \(0.58 \pm 0.18\)), but higher than normal (\(P < 0.05\)) in 5 untreated acromegalic patients (mean \(1.30 \pm 0.15\)). Pooled control serum from normal adult subjects was defined as having activity of 1 unit/ml of TIA. The mean TIA level of 6 untreated children with GH deficiency increased to normal (mean \(0.99 \pm 0.26\)) in the last month of treatment with human growth hormone (KABI-hGH), and that of 5 acromegalic patients decreased to normal or less (mean \(0.89 \pm 0.20\)) after transphenoidal hypophysectomy. There was a good correlation (\(r = 0.54, P < 0.001\)) between the TIA level and increase in height of 47 GH-deficient children treated with hGH. The TIA values in 22 blood samples obtained from 6 untreated children with GH deficiency and 16 GH-deficient children treated with hGH were well correlated with values of measurements of [\textsuperscript{3}H]thymidine incorporation into cultured growing chondrocytes of rabbits (\(r = 0.65, P < 0.001\)). These results suggest that serum activity to stimulate [\textsuperscript{3}H]thymidine incorporation into human fibroblasts has GH dependency, and should be a reliable index in bioassay of total somatomedin-like bioactivity in serum.

Radioreceptor assay (RRA) and radioimmunoassay (RIA) for somatomedin (Hall et al. 1974; Takano et al. 1975; Horner et al. 1978; Furlanetto et al. 1977) are now replacing bioassay techniques using cartilage (Daughaday et al. 1975), but the latter techniques are still necessary since values measured by RRA or RIA do not reflect biological activity. Somatomedin can stimulate both sulphate incorporation into cartilage (Salmon & Daughaday 1975) and thymidine incorporation into human fibroblasts and chick embryo fibroblasts (MacGillivray et al. 1975; Rechner et al. 1977; Cohen et al. 1975). The biological activities of these somatomedins have been determined mainly by measuring sulphate uptake into cartilage (Daughaday et al. 1975). Measurement of thymidine incorporation into human skin fibroblasts has not been generally used for assay of somatomedin activity in serum, because thymidine uptake into human skin fibroblasts was reported to be similar using sera from GH-deficient children before and after hGH-treatment and thus the method was concluded not to be accurate for measuring somatomedin activity (Moses et al. 1978).

This study was designed to evaluate the value of measuring thymidine incorporation into human skin fibroblasts in comparison with that into rabbit chondrocytes, and to find out whether values measured by this method correlate with the GH level. Activity for incorporation of thymidine into human fibroblasts is abbreviated as TIA in this report.

Materials and Methods

1) Cell culture

Fibroblasts were obtained from forearm skin biopsies of normal volunteers. The biopsies, \(2 \times 2 \times 2\) mm in size,
were put into 60 mm diameter plastic dishes (Falcon) and incubated in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS) (Flow Lab.). Fibroblasts were grown in a high humidity atmosphere of 5% CO₂ in air at 37°C until they became confluent. They were then passaged at a split ratio of 1:3 or 1:4, and the initial confluent monolayers were designated as the first cell population doubling (CPD). Cells were used for assays at passage numbers of 6 to 14 CPD. Growing cartilage cells were obtained aseptically from the cartilage junction of the ribs of young male New Zealand rabbits (400–700 g). The isolated chondrocytes were washed in Ham’s F-12 medium (Nissui Pharmaceutical Co., Tokyo) containing 10% FCS. Inocula of 0.5 ml of suspension containing 4 × 10⁴ cells were placed in each well of a Linbro tissue culture plate (Flow Lab.) and the cells were grown to confluence, as described in detail elsewhere (Kato et al. 1978, 1980).

2) Assay procedure

Fibroblasts (1 × 10⁴ cells) were incubated in 35 mm diameter plastic dishes and were cultured in MEM containing 10% FCS. After 24 h, the medium was replaced by serum-free MEM for 48 h. The medium was then replaced by MEM containing 5% test serum or control medium. After 18 h of exposure to the test media, the cells were pulsed for 1 h with [³H]thymidine (1 μCi/ml) in MEM. The radioactivity was counted by a modification of the methods of Jones & Addison (1976) and Moses et al. (1978).

Growing chondrocytes (primary cells) were used when they became confluent. The procedures used for starvation and stimulation of cell growth and pulse labeling were as for human fibroblasts.

In preliminary experiments, labelled fibroblasts were washed with PBS and fixed in ethanol. Acid-soluble materials were removed by washing the cells with 5% trichloroacetic acid for 1 h at 4°C. The fixed cells were washed with water for 30 min, and then the autoradiographic technique was applied. Nuclei in which [³H]thymidine was incorporated into DNA were densely labelled. The preparations were then stained with Giemsa (NERCK). Cells in which [³H]thymidine was incorporated into nuclei were called ‘positive nuclei’. The labelling index in our study was defined as the percentage of positive nuclei in 1200 cells. Details of the procedure are described elsewhere (Nakazawa et al. 1980).

3) Subjects

Sixty-eight GH-deficient children (15 female, 53 male) aged 4 to 21 years were studied. TIA was measured in 18 children before treatment with hGH and in the other 50 during treatment. The 50 GH-deficient children were treated with KABI-hGH (Crescormon®) at a mean dose of 0.21 ± 0.05 (mean ± sd) IU/kg/week for a mean period of 3.2 ± 1.1 (mean ± sd) years. They consist of 13 children with selective GH deficiency, and 37 with GH and gonadotrophin deficiency. Diagnostic criteria for hGH deficiency were described elsewhere (Okada et al. 1979).

The 60 non-GH-deficient children of short stature examined were 40 pre-pubertal and 10 pubertal children with constitutional short stature, and 12 patients with Turner’s syndrome. Five patients with anorexia nervosa, aged 14 to 21 years, were also studied as typical examples of a disease of malnutrition. TIA was measured in 6 acromegalic patients (3 female, 3 male), in 4 before and after trans-sphenoidal hypophysectomy (Hardy’s operation), in one before operation and in one after operation.

For comparison, TIA was also measured in 13 normal children aged 8 to 15 years. Pooled sera were obtained from 5 young men aged 25 to 30 years. Blood samples were drawn from the cubital vein of fasting subjects and the sera stored aseptically at −20°C for later analysis of TIA. In children treated with hGH, blood samples were taken at least 3 days after the last injection of hGH.
Results

1) Control serum
TIA was measured in five samples of pooled serum. The variation between the 5 samples was less than 10 per cent. The mean \(^{3}H\)thymidine incorporation (dpm) of the 5 samples was defined as 1 unit/ml, the normal range being 0.8 to 1.2 unit/ml. Each sample was assayed in duplicate. Fig. 1 shows that the \(^{3}H\)thymidine incorporation increased linearly when the dose of serum added to the incubation medium was increased from 0.5 to 10 per cent.

2) Comparison of TIA levels measured by autoradiography and by determination of total \(^{3}H\)thymidine incorporation into fibroblasts
To confirm that the \(^{3}H\)thymidine radioactivity incorporated into fibroblasts reflected DNA synthesis in the nuclei of the fibroblasts, we assayed TIA in 14 blood samples obtained from 5 cases of acromegaly, 5 of GH deficiency treated with hGH, and 4 of anorexia nervosa by measuring total radioactivity in a liquid scintillation counter and the labelling index by autoradiography. A good correlation \((r = 0.67, P < 0.01)\) was found between the values of TIA measured by the two methods, as shown in Fig. 2.

3) Comparison of TIA levels measured using human fibroblasts and rabbit growing chondrocytes
To determine whether the TIA values measured using human fibroblasts were correlated with the biological activity stimulating DNA synthesis in growing chondrocytes, we assayed 22 samples obtained from GH-deficient children before and after treatment with hGH using fibroblasts and chondrocytes. As shown in Fig. 3, a good correlation \((r = 0.65, P < 0.001)\) was obtained between the levels of TIA measured by these two procedures.

4) TIA in children of short stature
The mean TIA level was 0.63 ± 0.14 U/ml in 18 untreated GH-deficient children, 0.98 ± 0.21 U/ml in 50 treated cases, 1.05 ± 0.28 U/ml in 40 pre-pubertal children, 1.06 ± 0.18 U/ml in 10 pubertal children of short stature, 0.87 ± 0.24 U/ml in 12 patients with Turner's syndrome, and 1.04 ± 0.23 U/ml in 13 normal children. The TIA level was significantly \((P < 0.001)\) lower in untreated GH-deficient children than in groups of GH-deficient children treated with hGH, pre-pubertal constitutionally short children, pubertal constitutionally short children, children with Turner's syndrome, and normal children (Table 1). Four boys and 2 girls with GH deficiency aged 6 to 16 years were treated with hGH at doses of 0.15 to 0.34 IU/kg/week for a period of 6 months. TIA values were measured before and 2, 4 and 6 months after beginning of the treatment. The mean TIA levels in these children increased significantly \((P < 0.001)\) from 0.52 ± 0.09 U/ml to 1.04 ± 0.24 U/ml, 1.14 ± 0.30 U/ml and 0.97 ± 0.13 U/ml, respectively, during treatment. The TIA level of treated children with GH deficiency ranged from 0.71 to 1.38 U/ml. Individual height increase in the one year period before the test was correlated \((r = 0.54, P < 0.001)\) with the TIA level as shown in Fig. 4. No significant correlation was found between the dosage of hGH and the TIA level.

We also examined the correlation between TIA and height-increase in the one year period before
Correlation between thymidine incorporation activity (TIA) measured using human fibroblasts and growing chondrocytes (G.C.) in serum from 6 GH-deficient children and 16 GH-deficient children treated with hGH. Points are means for duplicate dishes.

the test in 26 GH-deficient children (4 pubertal and 22 pre-pubertal) who had been treated for 3 years or more. We divided these patients into two groups: a group of responders, whose height had increased 5 cm or more in the one year period before the test, and a group of non-responders, whose height had increased less than 5 cm in the previous year. The TIA of 12 responders ranged from 0.87 to 1.38 U/ml (1.04 ± 0.17 U/ml) and was significantly \((P < 0.05)\) higher than that of 14 non-responders, which ranged from 0.71 to 1.13 U/ml (0.90 ± 0.13 U/ml).

5) **TIA in acromegaly**

The TIA level ranged from 1.11 to 1.53 U/ml in untreated acromegalic patients and from 0.62 to 1.01 U/ml in 5 treated acromegalic patients. The mean TIA decreased significantly \((P < 0.05)\) from 1.34 ± 0.15 to 0.95 ± 0.19 U/ml after trans-sphenoidal hypophysectomy.

6) **TIA in anorexia nervosa**

In 5 patients with anorexia nervosa, the mean TIA level was 0.57 ± 0.18 U/ml, which was significantly \((P < 0.001)\) lower than that of control subjects. In two patients with anorexia nervosa, serial changes in TIA were studied during iv hyperalimentation (ivH). In one patient, ivH was carried out for 6 weeks; the calory intake was initially 1200 cal/day and was gradually increased to 1900 cal/day. The body weight of this patient increased from 30.0 to 31.5 kg during this treatment. The TIA was initially 0.34 U/ml and increased to 1.10, 0.63 and 1.41 U/ml after 2, 4 and 6 weeks treatment, respectively.

In another patient given ivH for 4 weeks, the calory intake was initially 1200 cal/day and was gradually increased to 2000 cal/day. Her body weight increased from 34.0 to 36.0 kg by the end of treatment. Her TIA was initially 0.44 U/ml and increased to 0.73, 0.50 and 0.79 U/ml in week 2, 3 and 4, respectively. Details of the procedure were described by Nakazawa et al. (1980).

### Discussion

The TIA of GH-deficient children was low before treatment, but increased to the normal range during treatment, while that of acromegalic patients was statistically high before trans-sphenoidal hypophysectomy and decreased to the normal range, or less, after the operation. Furthermore, the TIA in non-GH-deficient children of short stature did not differ statistically from that of control subjects or

<table>
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<tr>
<th>Subjects</th>
<th>n</th>
<th>Thymidine incorporation activity (TIA)</th>
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<tbody>
<tr>
<td>Normal children</td>
<td>13</td>
<td>1.04 ± 0.25</td>
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<tr>
<td>GH-deficient children</td>
<td>18</td>
<td>0.63 ± 0.14*</td>
</tr>
<tr>
<td>GH-deficient children treated</td>
<td>50</td>
<td>0.98 ± 0.21</td>
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<tr>
<td>with hGH</td>
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<td>Pre-pubertal constitutionally</td>
<td></td>
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<tr>
<td>short children</td>
<td>40</td>
<td>1.05 ± 0.28</td>
</tr>
<tr>
<td>Pubertal constitutionally</td>
<td></td>
<td></td>
</tr>
<tr>
<td>short children</td>
<td>10</td>
<td>1.06 ± 0.18</td>
</tr>
<tr>
<td>Turner's syndrome</td>
<td>12</td>
<td>0.87 ± 0.24</td>
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Values are means ± sd. *\(P < 0.001\) vs. normal children.
GH-deficient children treated with GH. In children with GH deficiency, individual TIA values correlated with the growth rate in the year before the test. These results suggest that the TIA measured with human skin fibroblasts depends on GH and reflects somatomedin-like bioactivity. Somatomedins, GH-dependent cell growth factors, are known to consist of somatomedin A, somatomedin C, insulin-like growth factor (IGF)-I, IGF-II and multiplication stimulating activity (MSA). The TIA measured by our method probably represents the sum of the biological activities of these somatomedins. In a preliminary experiment, we found that the biological activity of thymidine incorporation into the nuclei of human fibroblasts correlated with that of total radioactivity in cells. Thus TIA measured using human fibroblasts and growing rabbit chondrocytes indicates that our method measures biological activity to stimulate DNA synthesis not only in human fibroblasts but also in growing chondrocytes. Synthesis of mucopolysaccharide in the costochondral growth plate is known to be stimulated by rat serum (Hill 1979). Jennings et al. (1980) reported recently that purified somatomedin A, IGF-I and II enhanced [3H]thymidine incorporation, but not $^{35}$SO$_4^{2-}$ incorporation, into chick embryo pelvic leaflets. We also could not obtain a linear dose-response between the amount of serum and $^{35}$SO$_4^{2-}$ incorporation (data not shown), when synthesis was monitored by measuring incorporation of $^{35}$SO$_4^{2-}$ by the procedure of Kato et al. (1980). The results of our studies on thymidine incorporation into fibroblasts, however, were not consistent with the report of Moses et al. (1978) that thymidine incorporation into human fibroblasts of untreated GH-deficient children did not differ from that of treated ones. Because of this finding, they were forced to switch from human fibroblasts to chick embryo fibroblasts for the assay. Their method differed from ours in two respects: They used a period of cell starvation of 5–7 days and their incubation medium contained 0.4% FCS. Using their reported method, we found that the TIA levels of the sera of hGH-treated children were in fact not significantly higher than those of untreated GH-deficient children. Thus it seems likely that their starvation period is too long and that addition of 0.4% FCS to the medium decreased the sensitivity of cells in culture. Their sampling procedure also differed from ours; they examined namely the effect of hGH administration to GH-deficient children, for 1 or 2 days, whereas we examined the effect of treatment for 6 months. We cannot comment on the effect of this difference, since we did not examine the change of the TIA level after short-term hGH administration.

As confirmed by other investigators, the difference in the TIA levels of normal subjects and acromegalic patients was less than those of somatomedin C RIA and somatomedin A RRA (Furlanetto et al. 1977; Takano et al. 1975, 1976). A statistically significant difference was noted in the TIA levels of normal subjects and acromegalic patients, but the difference was less than that between normal subjects and GH-deficient children. The mean ratio of TIA in cases of acromegaly to that in GH-deficient children was 2:1, but this is a common result observed in bioassay (Hall 1970). RRA for somatomedin A and RIA for somatomedin C are specific methods for measuring somatomedin A and somatomedin C, respectively, in the serum and the levels measured by these methods reflect the clinical state (Clemmons et al. 1979), but do not indicate the biological activities of
these compounds. It is concluded that the measurement of TIA using human skin fibroblasts is a reliable method for assay of somatomedin-like bioactivity in the serum and that, since it is reliable, such a procedure is as useful as RIA and RRA.

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References


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