Increased Ferritin Levels in the Fluid of Thyroid Cyst

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Abstract

High ferritin levels in the aspirate of thyroid cyst (Six yellow clear, 4 yellow turbid and 10 chocolate colored turbid) without apparent sings of malignancy were found. The mean concentration in the 3,000×g supernatant of the fluid was 40,116 ng/ml, and the 3,000×g precipitate was 11,147 ng/ml. All cases showed normal levels of serum ferritin. Con A binding with ferritin was distributed from low to high. These ferritins showed a molecular weight of approximately 450,000 which was the same as found in human spleen and liver.

The continuous increase in ferritin levels in thyroid cyst fluid was found by a chronological study in some cases.

When tumor markers such as CEA, NCA, CA 19-9 and α_1 -acid glycoprotein (α_1 -AG, acute phase reactant) were examined simultaneously, an increase in some of the cyst fluid was observed. However, the incidence and the rate of increase of these tumor markers and acute phase reactant were low compared to ferritin. Neither a correlation between ferritin and CEA, nor between ferritin and CA 19-9 was found.

The increase in ferritin in thyroid cyst fluids may be due to the increased synthesis, release and storage by the inflammatory cells.

Ferritin is an iron storage protein which is present in liver, spleen, heart and other tissues (Walters *et al.*, 1973; Jacobs *et al.*, 1975). The ferritin molecule is composed of a protein shell and iron core, and has a molecular weight of 450,000 (Jacobs and Worwood, 1976).

The development of radioimmunoassay (RIA) has made possible the measurement of infinitesimal serum protein levels. The

measurement of the ferritin with RIA was developed by Addison *et al.* (1972). The measurement of serum ferritin levels has been considered as a useful marker for the detection and monitoring of malignant diseases by some investigators (Niitsu and Urushizaki, 1978), although ferritin is not a tumor specific index.

Recently, we found high ferritin concentrations in the aspirate of thyroid cyst, and investigated the properties of ferritin in the cyst fluid. This report describes the

experimental results including estimation of other tumor associated antigens, acute phase reactant and thyroglobulin in thyroid cyst fluids.

Materials and Methods

1) Samples

Aspirates of thyroid cyst and sera in 20 patients (1 man, 19 women) with thyroid cyst without malignancy were used. There were 6 yellow clear, 4 yellow turbid and 10 chocolate colored turbid fluids. Aspirates of thyroid cyst were divided into the supernatant and precipitate by centrifugation at $3,000 \times g$ for 30 min. The precipitate was resuspended in a volume equal to that of the supernatant by adding 0.1 M phosphate buffer pH 7.8. These preparations were frozen at -20°C until use. After thawing, they were centrifuged at $25,000 \times g$ for 60 min at 4°C , and the supernatant was used for RIA.

Five patients were investigated chronologically for changes in ferritin levels in cyst fluids of thyroid. Four thyroid tissues were obtained at operation from patients with hyperthyroidism. The tissue was homogenized in 0.1 M phosphate buffer, pH 7.8. Fifty percent (w/v) thyroid homogenate was centrifuged at $25,000 \times g$ for 60 min at 4°C . The supernatant was used for RIA.

Sera were obtained from 10 volunteers (5 men, 5 women) who had mean ferritin levels of 82 ± 41 ng/ml (mean $\pm$ SD), and from 5 patients with cancer (2 lung cancer; 457 (mean), 1 liver cancer; 597, 1 pancreas cancer; 859 and 1 uterus cancer; 412) who had mean ferritin levels of 556 ng/ml. Tumor tissue of hepatoma was obtained at autopsy, then 20% (w/v) homogenate was centrifuged at $25,000 \times g$ for 60 min to obtain the supernatant.

2) Determination of ferritin and other protein levels

Ferritin (Travenol Co. Boston), CEA (Commissariat A L'energie Atomique CIS, France), CA 19-9 (CIS) and thyroglobulin (Eiken Lab. Tokyo, Japan) were measured using commercial RIA kits. Non-specific cross-reacting antigen (NCA) levels were also measured with the RIA method developed in our lab (Hamazu *et al.*, 1984). α_1 -acid glycoprotein (α_1 -AG) levels were

measured with commercial single radial immunodiffusion kits (MBL, Tokyo, Japan). The normal values were ferritin; <200 ng/ml, CEA; <10 ng/ml, CA 19-9; <37 U/ml, thyroglobulin; <20 ng/ml, NCA; <200 ng/ml and α_1 -AG; 30-95 mg/dl, respectively. Total proteins were determined by Lowry's method (Lowry *et al.*, 1951).

3) Estimations of thyroid function

Thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH) were measured by the methods reported previously (Ishida *et al.*, 1982). Normal values for T4, T3 and TSH were 5.1-11.2 $\mu\text{g/dl}$, 0.8-2.0 ng/ml and <5 $\mu\text{U/ml}$, respectively.

4) Gel-filtration on Sepharose 6B

One ml of the supernatant obtained from the aspirate (355,800 ng ferritin/ml) was fractionated on Sepharose 6B (Pharmacia, Uppsala) column (120×1.5 cm) in 0.05 M phosphate buffer, pH 7.8. These fractions were monitored at 280 nm for protein and collected in 2 ml aliquots. These fractions were used for ferritin determination. ^{125}I -labeled human spleen ferritin (CIS-RIA kit, about 100,000 cpm/ml) or ^{125}I -labeled human liver ferritin (Travenol-RIA kit, about 150,000 cpm/ml) was mixed with 0.5 ml of normal human serum (NHS), before fractionation on Sepharose 6B column.

5) Affinity chromatography with Con A-Sepharose

Each aspirate of thyroid cyst (100 μl) was mixed with 250 μl of Con A-Sepharose (Pharmacia, Uppsala) which had been equilibrated with 0.1 M acetate buffer (pH 6.0) containing 1 mM MgCl_2 , MnCl_2 and CaCl_2 , and these mixtures were allowed to stand for 24 hr at 4°C . The supernatant was obtained by centrifugation at 3,000 rpm for 30 min.

Results

1) Ferritin levels in the aspirate of thyroid cyst without malignancy

When ferritin level in 4 cases (No. 1-4) of yellow turbid fluid and 10 cases (No. 5-14) of chocolate colored turbid fluid was examined, the $3,000 \times g$ precipitate fraction showed a higher ferritin level than the 3,000

$\times g$ supernatant fraction. Six yellow clear fluids (No. 15-20) that had no precipitate fraction also showed high ferritin levels ($>1,000$ ng/ml) (Fig. 1). Mean ferritin levels were 40,116 ng/ml in the $3,000\times g$ supernatant fraction and 11,147 ng/ml in the $3,000\times g$ precipitate fraction (released fraction after freezing), respectively.

All patients' sera showed normal ferritin levels (<100 ng/ml). There was no correlation between serum ferritin levels and aspirate ferritin levels in patients with thyroid cyst (Fig. 2).

The mean level of ferritin in the $25,000\times g$ supernatant of thyroid tissues was 1,013 ng/ml (Table 1).

Fig. 1. Ferritin levels in the fluid of thyroid cyst. Each bar shows ferritin levels.

Fig. 2. Correlation between serum ferritin and ferritin in the $3,000\times g$ supernatant of aspirate.

2) CEA, NCA, α_1 -AG and CA 19-9 levels in the aspirate of thyroid cyst

CEA, NCA, α_1 -AG and CA 19-9 levels in the aspirate were examined. Two cases showed high CEA levels in the supernatant fraction (495 and 88 ng/ml).

Seven cases out of 13 patients (7/13) in the supernatant fraction and 6 cases out of 13 patients (6/13) in the precipitate fraction had high NCA levels (>200 ng/ml).

Out of 15 cases studied α_1 -AG amounts were high in 6 supernatants and in one precipitate. All cases except one showed trace amounts of α_1 -AG in the precipitate.

CA 19-9 levels were slightly elevated in 6 cases and extremely elevated in 6 cases in the supernatant (the normal range is <37 U/ml). However, only 5 cases showed high levels in the precipitate (Fig. 3).

3) Relationship between ferritin levels and other tumor markers (CEA and CA 19-9)

in the supernatant fraction of thyroid cyst fluids

The coefficient of correlation between ferritin and total proteins was 0.39 ($p < 0.05$). The coefficients of correlation between ferritin and CEA, and between ferritin and CA 19-9 were 0.04 ($p > 0.05$) and 0.02 ($p > 0.05$), respectively.

4) Thyroid function and clinical course in the patients with thyroid cyst

All patients with thyroid cyst showed normal serum T₄, T₃ and TSH levels. Aspirates of thyroid cyst in all patients were examined histologically, but no malignant findings were observed.

The chronological change in ferritin, CEA, NCA, α_1 -AG and CA 19-9 were examined in some patients with thyroid cyst. The clinical course of one patient is shown in Fig. 4. Ferritin levels in the thyroid cyst fluid of this patient were continuously

Table 1. Ferritin, thyroglobulin and total protein levels in thyroid cyst fluid and thyroid tissues

Preparation	Ferritin (ng/ml)	Thyroglobulin** (μ g/ml)	Total protein (mg/ml)
Thyroid cyst fluids			
Supernatant (n=20)	40,116	4,856	110.9
Precipitate (n=14)	11,147	445	67.7
Thyroid tissues* (n=4)	1,013	$>32,000$	107.6

*; The 25,000 $\times$ g supernatant was used for determination

**; Normal serum level was <27 ng/ml

Fig. 3. CEA, NCA, α_1 -acid glycoprotein and CA 19-9 levels in the fluid of thyroid cyst. Shaded area shows the normal range.

high. Other patients also showed a similar pattern (Data not shown).

5) *Thyroglobulin levels in the aspirate and thyroid tissues*

Thyroglobulin levels in the aspirate of thyroid cyst were high, especially in the supernatant. The mean level of thyroglobulin was 4,856 $\mu\text{g/ml}$ in the supernatant and 445 $\mu\text{g/ml}$ in the precipitate, respectively. Thyroglobulin levels in the 25,000 $\times$ g supernatant of thyroid tissues were extremely high ($>32,000 \mu\text{g}/107.6 \text{ mg protein}$) (Table 1).

6) *Gel-filtration pattern of ferritin in the thyroid cyst fluid using Sepharose 6B*

The elution pattern of thyroid cyst ferritin on Sepharose 6B is shown in Fig. 5. The peak of ferritin was found in the

molecular weight range of approximately 450,000. ^{125}I -labeled ferritin from human liver (Travenol) and human spleen (CIS) showed that the eluted radioactivity has a similar molecular weight (the elution pattern is shown in parenthesis).

7) *Con A binding with ferritin in the aspirate of thyroid cyst*

Experimental results of Con A binding of ferritin in the thyroid cyst fluid, normal human serum, cancer patient's serum, tumor tissue of hepatoma, and thyroid tissue in Graves' patients are shown in Fig. 6. The unbound percentage in the aspirate was widely distributed (the range was 6.8–90.7 %), although the mean percentage was 38.3 %. The mean unbound percentage in normal human serum and in cancer patient's serum was 20.1 % and 44.3 %, respectively.

Fig. 4. Time course of ferritin, CEA, NCA, α_1 -acid glycoprotein and CA 19-9 levels in the fluid of thyroid cyst. Shaded area shows the normal range.

The unbound percentage in tumor tissue of hepatoma and thyroid tissue was 65.4 % and 47.6 %, respectively. There was no correlation between ferritin levels and Con A binding of ferritin in the aspirate of thyroid cyst. However, the positive correlation between Con A binding of ferritin in the supernatant and the precipitate from the same sample was statistically significant ($r=0.89$, $p<0.01$).

Discussion

Recently, many tumor associated antigens have been available for the monitoring and as diagnostic tools in patients with malignant diseases. Some of them are CEA (Gold and Freedman, 1965), CA 19-9 (Koprowski *et al.*, 1979) and alpha-fetoprotein (Abelev *et al.*, 1963). Ferritin has been known as an iron storage protein and used for diagnosing iron deficiency anemia and iron overload. On the other hand, it was regarded as a tumor associated antigen (Marcus *et al.*, 1979) after

Fig. 5. Gel-filtration of the fluid in thyroid cyst using Sepharose 6B.
Vo; void volume,
Tg; thyroglobulin
Alb; albumin
The parenthesis shows the elution pattern of ^{125}I -liver or spleen ferritin containing NHS.

Fig. 6. Con A binding of ferritin in the fluid of thyroid cyst, normal human serum, cancer patient serum, tumor tissue of hepatoma and thyroid tissue. Each bar shows the mean value.

the development of RIA for ferritin by Addison *et al.* (1972). A high serum ferritin level in lung cancer (Gropp *et al.*, 1978), leukemia (Jones *et al.*, 1973), primary hepatoma (Kew *et al.*, 1978), and other malignant diseases (Niitsu *et al.*, 1975) has been reported.

In the present experiment we found high ferritin levels in the aspirate of thyroid cyst showing no apparent sign of malignant disease. As far as we know, this is the first report for a high ferritin level in the aspirate of benign thyroid cyst in spite of the fact that the level was normal in serum ferritin. High levels of CEA, CA 19-9 and α_1 -AG (one of acute phase reactants) were also observed in some thyroid cyst fluids. However, the frequency and rate of increase of these markers were significantly lower than those of ferritin. We could not compare these tumor markers in cyst fluid from cystic thyroid carcinoma in the present experiment. However, a significantly increased production of ferritin in cyst fluid of thyroids without malignancy was thought highly probable.

Interestingly, high ferritin levels were found not only in the $3,000 \times g$ supernatant of the fluid, but also the $3,000 \times g$ precipitate fraction that was released into the $25,000 \times g$ supernatant fraction after freezing. This fact means that ferritin in leucocyte and in macrophage may be released after freezing ($-20^\circ C$). The mechanism for increased ferritin levels in cyst fluid of thyroids may be participated in by many factors such as increased production in the inflammatory cells (yellow turbidity and chocolate colored turbidity may be due to an inflammatory mechanism), the increased release from necrotic tissue (this finding was obscure) and the decrease in the decomposition of ferritin in the cyst.

It has been reported that ferritin levels in synovial fluids were high in rheumatoid arthritis and low in osteoarthritis. This means that ferritin may be associated with

the reticuloendothelial cells which were stimulated by the inflammatory process (Blake *et al.*, 1980). Another report showed that ferritin production was higher in leukemia cells than in normal leucocytes (Worwood *et al.*, 1974), and that ferritin was detected in T-cells but not B-cells (Nishiya *et al.*, 1980).

An increase in serum ferritin levels in non-malignancy was shown in inflammation (Lipschitz *et al.*, 1974), and this may be the cause of increased ferritin synthesis (Konijn and Hershko, 1977).

The ferritin molecule has a heterogeneity. Linder *et al.* (1974) suggest that the ferritin molecule is composed of at least two types of subunits (H and L) and the proportion of these subunits varies with the iron load.

There are many reports on the binding of ferritin with Con A, because some ferritins contain carbohydrate moieties (Shinjo *et al.*, 1975). Worwood *et al.* (1979) found that only the acidic iso-ferritins of human serum bound to Con A while the most basic iso-ferritins did not. Halliday *et al.* (1979) reported that the plasma half-life of ferritin which was bound to Con A was approximately twice that of ferritin which did not bind. Birgegard (1980) showed that ferritin derived from tissue necrosis is unbound to Con A, but serum ferritin is usually bound to Con A. The present data also confirmed that tissue ferritin was unbound to Con A and serum ferritin was bound to Con A. This phenomena may be due to the fact that the most plasma proteins become glycosylated during secretion into the plasma. Such a process may be important in determining the properties of circulating ferritin (Worwood *et al.*, 1979).

Ferritin in cyst fluid of thyroid did not show constant binding with Con A. This may indicate that the synthesis and storage of ferritin in the thyroid cyst fluid may be participated by various mechanisms and factors.

Ferritin levels and total proteins in the

aspirate of thyroid cyst showed weakly positive correlation. The thyroglobulin levels in the aspirate of thyroid cyst was higher than the thyroglobulin level in normal serum, but lower than in thyroid tissue.

CA 19-9 was discovered by Koprowski *et al.* (1979) with hybridoma techniques, and this antigen is comparatively high in malignancy, especially in patients with pancreas or gastrointestinal tract cancer (Del Villano *et al.*, 1983).

In the present study, CA 19-9 levels in some cases of the aspirate of thyroid cyst were high although the frequency was low. CEA levels were also high in some cases in spite of the absence of a malignancy. Elevation of CEA, CA 19-9 and α_1 -AG was not as frequent as ferritin. These facts may indicate that increased tumor marker and acute phase reactant in the thyroid cyst fluid may be attributed to the production by the inflammatory cell. It is interesting to note that extremely high ferritin levels in cyst fluid of thyroids without apparent signs of malignancy are common, although plasma ferritin levels are normal. The phenomenon may be attributed not only to the production and release of large amounts of ferritin from inflammatory cells, but also to the decrease in the decomposition of ferritin and to the condensation of ferritin in thyroid cyst fluid.

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