Human Chorionic Gonadotropin-Like Substance in Nonendocrine Tissues of Normal Subjects

Abstract. *By means of two assay systems, a beta chain human chorionic gonadotropin radioimmunoassay and a radioreceptor gonadotropin assay, a chorionic gonadotropin-like substance was demonstrated in extracts of liver and colon obtained at autopsy from three patients who died of nonneoplastic disease. In contrast to placental chorionic gonadotropin, colon and liver chorionic gonadotropin was not bound to concanavalin A–Sepharose columns, indicating that this substance possessed little or no carbohydrate. Previous workers demonstrated that desialylated human chorionic gonadotropin possesses little or no bioactivity in vivo but retains full radioreceptor and radioimmunoassay activity in vitro. Our data suggest that the genome responsible for the human chorionic gonadotropin production is not completely suppressed in adult nonendocrine tissues, and that the chorionic gonadotropin produced by colon and liver has little or no bioactivity in vivo because of its low carbohydrate content. Since many normal tissues produce chorionic gonadotropin, bioactivity may be modulated by regulation of carbohydrate content.*

Human chorionic gonadotropin (CG) is normally secreted by the cytotrophoblast and syncytiotrophoblast of the placenta, by tumors developing from these cell types (gestational trophoblastic neoplasms), by teratomas of the ovary, testis, or pineal, by hepatoblastomas, and by 6 to 13 percent of carcinomas of the lung, stomach, pancreas, and colon (*1*). We have postulated that ectopic peptide elaboration is a universal concomitant of cancer and that this represents activation of repressed genic data during the process of neoplasia (*2*). Recently, Braunstein *et al.* (*3*) reported the presence of CG in extracts of normal human testes and indicated that extracts from other tissue contained no such substance. To evaluate further whether activation of the genome for CG synthesis is specific for cancer or, alternatively, also occurs during cell replication of other normal cells, we have studied extracts from tissues of patients who died without cancer. We report herein those data.

Normal liver and colon were obtained at the time of autopsy from three male patients who died of nonneoplastic disorders. The tissues were frozen at −70°C until used. They were then weighed, minced, and homogenized in a blender with 5 ml of normal saline solution per gram of tissue. The homogenates were agitated in a water bath at 85°C for 30 minutes and centrifuged at 10,000*g* for 30 minutes at 4°C. The supernatants were lyophilized and reconstituted in 0.01*M* phosphate-buffered saline (PBS), *p*H 7.4, at the equivalent concentrations of 3 g of unextracted colon tissue and 6 g of unextracted liver tissue per milliliter. These were again centrifuged at 15,000*g* for 30 minutes. Portions of the supernatants were subjected to radioimmunoassay and radioreceptor assay. As a separate control, human CG (Antuitrin-S, Parke, Davis) was added to the liver and colon tissues to assess recovery during extraction. Recovery was 49 percent for liver extracts and 97 percent for colon.

Rat testicular homogenates for the radioreceptor assay were obtained according to the method of Catt and co-workers (*4*). That is, testes removed from adult Sprague-Dawley rats were decapsulated, minced in 4 ml of cold Krebs-Ringer bicarbonate buffer (*p*H 7.4) per gram of tissue, and homogenized. After filtration over eight layers of gauze, the suspension of separated tubules was centrifuged at 120*g* for 20 minutes. The supernatant solutions were then centrifuged at 1500*g* for 20 minutes and the pellets were resuspended in 0.01*M* PBS, *p*H 7.4 (3 ml per gram of tissue, wet weight). Both intact pituitary luteinizing hormone (LH) and CG give identical dose response curves in this radioreceptor assay; the free β chain of CG does not react in this assay.

A modification of the chloramine-T method was used to label the CG or the β subunit of CG with radioactive iodine. Reagents were added (at 4°C) to the iodination vial in the following order: 50 μl of 0.5*M* PBS, *p*H 7.4; 1 mc of ^{125}I (New England Nuclear); 2 μg of CG or CG-β in 10 μl of 0.01*M* PBS; 2.5 μg of chloramine-T in 5 μl of 0.01*M* PBS. After the mixture had been shaken gently for 20 seconds, the reaction was terminated by the addition of 300 μl of 0.01*M* PBS containing 1 percent bovine serum albumin (BSA). The mixture was then transferred to a column (8.5 by 50 mm) of Sepharose–concanavalin A (Con A) (Pharmacia) which was washed extensively with 1 percent BSA in PBS. Free iodine and damaged hormone were eluted with 20 ml of PBS, and the labeled hormone was then eluted with the same solution containing 0.2*M* methyl-αD glucopyranoside (Calbiochem).

The CG was measured by a double antibody radioimmunoassay with the following reagents (*5*): purified CG (CR-119), the β subunit of CG (β subunit of CG CR-115) that was used for iodination and as the reference standard, and antiserum to the β subunit of CG-SB6. The lower limit of sensitivity of this assay was 250 pg per assay tube. The intra-assay coefficient of variation (calculated from duplicates) averaged 12.4 percent, and the interassay coefficient of variation was less than 20.8 percent. In this beta CG radioimmunoassay, pituitary LH, CG, and the β chain of CG all react.

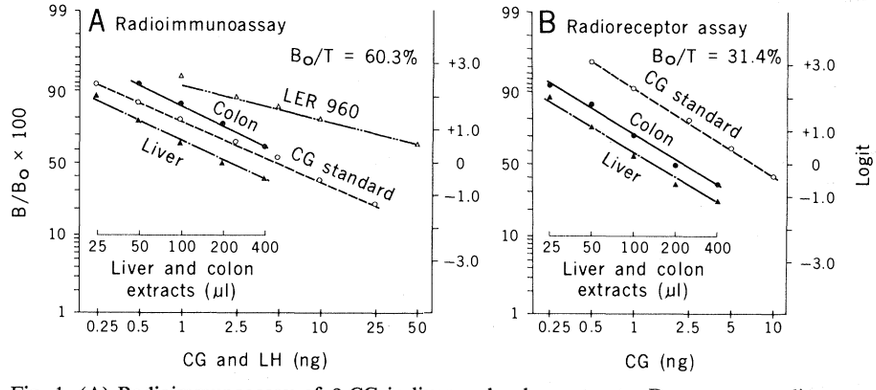

Fig. 1. (A) Radioimmunoassay of β-CG in liver and colon extracts. Dose response lines are shown for the CG reference preparation (standard) and for liver and colon extracts. Results are shown as the log dose of the CG standard or of the volume of liver and colon extracts added per tube plotted against the logit transformation of the response. B_0 is the counts per minute bound to antibody or receptor when no unlabeled CG was present in the tube. B indicates the counts per minute bound when unlabeled CG was competing with the ^{125}I-labeled CG. T is the total count per minute per tube. (B) Radioreceptor assay for CG-LH. Dose response lines are shown for the CG reference preparation (standard) and the liver and colon extracts. Results are shown as the log dose of the CG standard or of the volume of liver or colon extracts added per tube plotted against the logit transformation of the response. B_0 and B are defined as in (A).

However, LH and CG (and the β chain of CG) give different dose response curves and may be thus distinguished. Human LH has less than 10 percent of the potency of CG in this assay.

For the radioreceptor assay the method of Catt and co-workers was used. Receptor-bound CG was separated from free CG by centrifugation at 1500*g* for 30 minutes at 4°C; the supernatants were aspirated. After a single wash with 3-ml portions of cold PBS, the tubes were recentrifuged and the supernatant aspirated. The radioactivity in the sediments was counted in an automatic gamma spectrometer. The minimum detectable value was 1 ng of CG per assay tube. Statistical analyses both in the radioimmunoassay and the radioreceptor assay were made according to the method of Bliss (*6*).

Affinity chromatography with Con A was performed at room temperature as follows. Columns (8.5 by 50 mm) of Sepharose–Con A were prepared and washed with 0.01*M* PBS containing 1 percent BSA. Portions (3 ml) of liver or colon extracts were applied to the columns and eluted with 10 ml of 1 percent BSA in PBS. This was followed by a second elution with PBS containing 0.2*M* methyl-α-D-glucopyranoside. Fractions were collected in 1.0-ml portions in glass tubes containing 50 μl of 1 percent BSA in PBS. The recovery of CG activity that had been present in the tissue extracts, or of ^{125}I-labeled CG and ^{125}I-labeled CG subunit that had been added to the column, ranged from 74 to 87 percent.

Serial dilutions of normal human liver and colon extracts had dose response curves that were parallel to the CG reference standard both in the radioimmunoassay and the radioreceptor assay (Fig. 1). These extracts contained 25 to 40 mg of protein per milliliter (as determined by the Lowry method). Control studies revealed that the addition of 20, 40, and 60 mg of protease-free BSA per assay tube, in addition to the 1 percent BSA in the assay buffer, had no effect on the binding of ^{125}I-labeled CG in either type of assay. In the radioimmunoassay and the radioreceptor assay, respectively, the colon extract contained 3.3 and 11.7 ng of CG (ratio of 3.5), and the liver extract contained 4.1 and 8.3 ng of CG activity (ratio 2.0), per gram of tissue. When the colon extracts were studied by means of the Sepharose–Con A columns, the CG did not bind to the column. Thus, by the radioreceptor assay and the radioimmunoassay, respectively, 96 and 89 percent of the CG in the colon extracts were eluted in the void volume, whereas only 4 and 11 percent of the CG bound to the column and were eluted with 0.2*M* methyl-α-D-glucopyranoside (MαGP) (Fig. 2A). A similar profile was observed for liver extract (Fig. 2B). Rechromatography of the combined (tubes 3 and 4) fractions of liver extract (see Fig. 2B) which were not adsorbed on Con A, again showed that all the CG was in the void volume (Fig. 2C). In contrast to the colon and liver CG, 90 percent of the placenta CG that was prepared identically bound to Con A and was eluted by 0.2*M* MαGP. Tissue extracts from all three subjects produced similar results. To evaluate whether colon and liver tissues might remove carbohydrate from CG after synthesis, we incubated placental CG with fresh homogenates of liver and colon for 2 hours at 37°C. No change in the binding with Con A resulted, indicating the carbohydrate had not been removed.

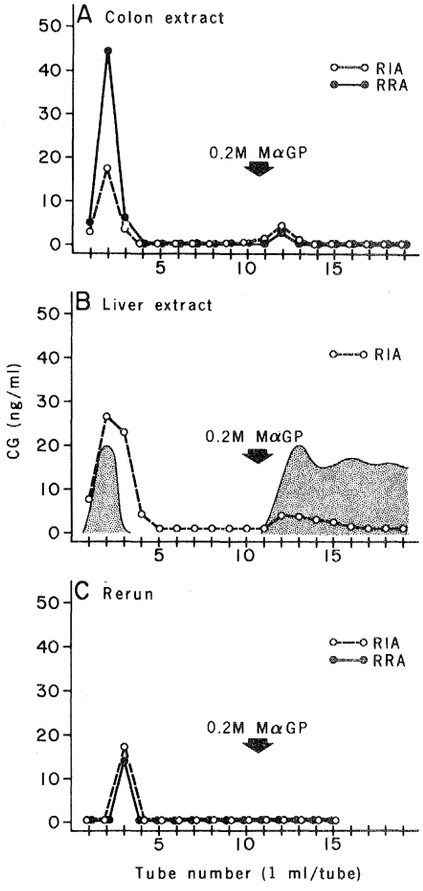

Fig. 2. Affinity chromatography on a column of Sepharose–Con A of (A) colon extract, (B) liver extract, and (C) a rerun of the combined fractions (tubes 3 and 4) of liver extract in (B). The void volume of this column by blue dextran was 0.75 ml. Four to 14 percent of the CG in both the colon and liver extracts, measured by radioimmunoassay (RIA) and radioreceptor assay (RRA), were eluted with methyl-α-D-glucopyranoside (MαGP). In (B) the shaded area indicates the elution profile of extracts of normal placenta. The amount of CG in this placental extract applied to the column was 220 ng as determined by radioimmunoassay. In contrast to liver or color CG, only a small fraction of the placental CG was eluted before the addition of MαGP.

These data indicate that a substance is present in normal colon and liver extracts which reacts in the β-CG radioimmunoassay and in the LH-CG radioreceptor assay. Since free β chain of CG does not react in the radioreceptor assay, and LH and CG give different slopes in the radioimmunoassay, this material in colon and liver is probably intact chorionic gonadotropin. In addition, Van Hall *et al.* (*7*) and Tsuruhura *et al.* (*8*) have shown that desialylated CG possesses increased potency in the radioimmunoassay, full potency in the radioreceptor assay, but extremely little (the exact value depends on the bioassay used) bioactivity when measured in vivo. This loss of bioactivity in vivo and maintenance of bioactivity in vitro was shown to be related to an enhanced metabolic clearance; that is, the substance rapidly disappeared from the circulation. While the CG-like material of colon and liver extracts reacts in vitro in both assays, it does not bind to Con A, indicating little or no carbohydrate is present. Accordingly, we conclude that this CG-like material has the peptide structure of CG but does not possess the carbohydrate moieties, and probably has little or no bioactivity in vivo. Furthermore, the peptide structure may be synthesized without the carbohydrate moieties, since colon and liver extracts did not remove carbohydrate from placental CG. Recently, Chen *et al.* (*9*) demonstrated the presence of CG-like material in pituitary and urinary extracts of a patient with Klinefelter's syndrome. These data also support the concept that normal tissues produce the peptide structure of CG.

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Mutagenic Activity of Quercetin and Related Compounds

Abstract. *The mutagenic activities of several flavonoids and flavonoid metabolites were examined by means of* Salmonella typhimurium *mutants that reveal base-pair substitution and frameshift mutagens. Of the compounds tested (naringin, rutin, neohesperetin, hesperetin, dihydroquercetin, quercetin, quercetin pentaacetate, permethylquercetin,* m-*hydroxyphenylacetic acid, and* m,p-*dihydroxyphenylacetic acid), only quercetin was mutagenic without microsomal activation. With activation, however, the mutagenic activity of quercetin was increased significantly and that of quercetin pentaacetate was revealed. The health implications of these findings and aspects of flavonoid structural requirements for mutagenic activity are discussed.*

Quercetin (5,7,3′,4′-tetrahydroxyflavone) is one of the most common phenolic compounds in vascular plants (*1*). It occurs in conjugated or free form in many plant products used for food, including many fruits, vegetables, and tea (*2*). The chemical and biological activities of many flavonoids, including quercetin, have been the subject of extensive study for many years (*3*). There is also considerable interest in the flavonoid derivatives as possible nonnutritive sweeteners (*4*).

Considering the widespread occurrence of quercetin in foods, relatively little work has been done on the toxicity of this compound. Ambrose *et al.* (*5*) found a low toxicity for quercetin in short-term and long-term (410 days) studies with rabbits and rats. Subsequent studies by Busby *et al.* (*6*) in which quercetin was implanted in bladders of mice indicated that quercetin might have tumorigenic activity. We have examined the mutagenic activity of quercetin and some related compounds using *Salmonella typhimurium* mutants to detect mutagenicity as described by Ames *et al.* (*7*).

Four *S. typhimurium* strains were used in this study: TA1535 and TA100 which carry the allele *hisG46* that is reverted by base-pair substitutions, and TA1538 and TA98 which carry the *hisD3052* allele that is reverted by frameshift mutations. Strains TA100 and TA98 carry the plasmid R factor pKM101, but TA1535 and TA1538 do not. Mutagenicity of substances is determined by incubating them with the bacterial strains and noting the number of histidine-independent (revertant) clones. In a parallel series of experiments the test substances and bacteria are incubated with a liver microsome preparation. An increased number of revertants under these conditions indicates a metabolic enhancement of mutagenic activity.

The flavonoids were obtained from commercial sources and derivatives were prepared synthetically by conventional procedures. All compounds were obtained in crystalline, analytically pure form and were used as solutions in dimethylsulfoxide in the mutagenicity tests.

Naringin, rutin, neohesperetin, hesperetin, quercetin, dihydroquercetin, quercetin pentaacetate, permethylquercetin, and two quercetin metabolites, *m*-hydroxyphenylacetic acid and *m,p*-dihydroxyphenylacetic acid, were assayed against TA98 and TA100 by the spot (100 μg per disk) and plate (50 μg, 250 μg, 1.0 mg, and 2.5 mg per plate) incorporation methods (*7*). Mutagenic activities of quercetin and quercetin pentaacetate were observed against both strains. Quercetin pentaacetate required metabolic activation for mutagenicity, whereas quercetin did not. The other compounds tested were not active against these strains. Corresponding experiments in which quercetin, dihydroquercetin, quercetin pentaacetate, permethylquercetin, *m*-hydroxyphenylacetic acid, and *m,p*-dihydroxyphenylacetic acid were assayed against TA1538 and TA1535 revealed mutagenic activity of quercetin against TA1538. Other compounds in this series were not active against TA1538 and no compound showed mutagenic activity against TA1535.

More detailed analysis of quercetin and quercetin pentaacetate revealed (Fig. 1) obvious dose-related responses for TA98 and TA100 and a less pronounced but definite relationship for quercetin when tested against TA1538.

Quercetin exhibits frameshift mutagenic activity by inducing reversions of the frameshift mutation in strains TA98 and TA1538. Like most frameshift mutagens, quercetin has a planar, polycyclic structure. Presumably, it acts by intercalating in the DNA base stack and inducing mispairing in a string of nucleotides. When such a mispaired DNA sequence is replicated, there is a greatly increased probability of adding or deleting a base pair in the nascent sequence and thus generating a frameshift mutation (*8*).

Molecular planarity appears to be a requirement for mutagenic activity in the compounds tested since the nonplanar flavonoids with a reduced pyrone ring, hesperetin, naringin, and most notably, dihydroquercetin, exhibit no measurable activity. However, since the planar compound, permethylquercetin, does not exhibit mutagenic activity, a requirement for free phenolic groups is also suggested. Indeed, whereas the planar com-

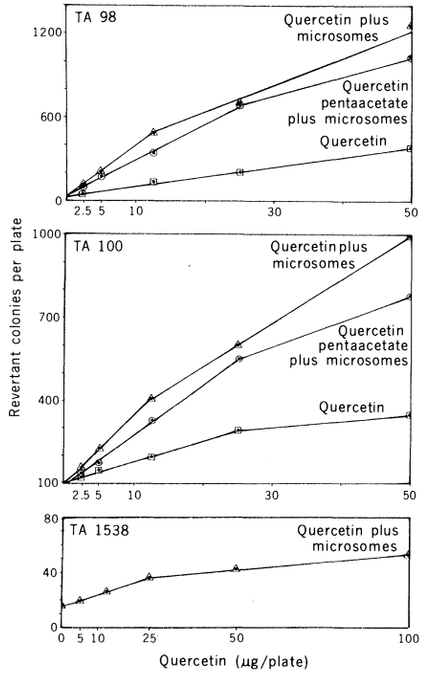

Fig. 1. The number of revertant colonies of the indicated strains as a function of the dose of mutagen. Each point is the result of a count of a separate plate. Spontaneous reversions are included with each count. The procedure was that described in (*7*), and 100 μl of liver preparation previously induced with phenobarbital was added to the appropriate plates.