Influence of TSH on uptake of [18F]fluorodeoxyglucose in human thyroid cells in vitro

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Abstract. Recent clinical evidence suggests that positron emission tomography with fluorine-18 fluorodeoxyglucose (FDG-PET) is more accurate in detecting thyroid carcinomatous tissue at high than at low TSH levels. The aim of this study was to determine the influence of TSH on FDG uptake in human thyroid cells in vitro. Monolayers of human thyroid tissue were cultured after mechanical disintegration and enzymatic digestion of samples from patients undergoing surgery for nodular goitre. The purity of thyroid cell preparations was ascertained by immunohistochemical staining for the epithelial antigen KL-1, and their viability by measuring the synthesis of thyroglobulin in vitro. The cells were incubated with 0.8–1.5 MBq FDG/ml uptake medium for 1 h. FDG uptake in thyroid cells was quantified as percent of whole FDG activity per well (% ID) or as % ID in relation to total protein mass. This experimental protocol was subsequently varied to study the effect of incubation time, glucose dependency and TSH. Furthermore, radio-thin layer chromatography was used to identify intracellular FDG metabolites. FDG accumulated in the thyroid cells linearly with time, doubling roughly every 20 min. Uptake was competitively inhibited by unlabelled glucose and decreased to approximately 70% at 100 mg/dl glucose compared to the value measured in glucose-free medium. FDG was intracellularly trapped as FDG-6 phosphate and FDG-1,6-diphosphate. TSH significantly increased FDG uptake in vitro in a time- and concentration-dependent manner: Cells cultured at a TSH concentration of 50 µU/ml doubled FDG uptake compared to TSH-free conditions, and uptake after 72 h of TSH pre-incubation was approximately 300% of that without TSH pre-incubation. TSH stimulates FDG uptake by benign thyroid cells in a time- and concentration-dependent manner. This supports the clinical evidence that in well-differentiated thyroid carcinomas, most of which are still TSH-sensitive, FDG-PET is more accurate at high levels of TSH.

Keywords: FDG – PET – TSH – Thyroid – Thyroid carcinoma

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Introduction

Thyroglobulin (Tg), as a specific product of functionally differentiated thyrocytes, is an accurate tumour marker in the clinical follow-up of thyroidectomised patients with differentiated thyroid carcinoma. The elevation of its concentration in patients' sera sensitively and specifically reflects progression of differentiated thyroid cancer at high TSH levels [1, 2]. When Tg levels indicate the onset of recurrence, it is crucial to identify the site of Tg production. The procedure of first choice is whole-body scintigraphy after administration of iodine-131. However, a frequently observed complication in progressing thyroid carcinoma is loss of the ability of metastases to concentrate iodine. This makes the recurrence undetectable and also untreatable by radioiodine, although this is a controversial issue [3, 4].

For the scintigraphic detection of iodine-negative metastases, several alternative radiopharmaceuticals suitable for single-photon emission tomography (SPET) are in clinical use, e.g. thallium-201 and technetium-99m sestamibi [5, 6, 7]. However, although these tracers are well established in the workup of patients with thyroid cancer, positron emission tomography with fluorine-18 fluorodeoxyglucose (FDG-PET) is increasingly being performed to localise radioiodine-negative metastases of differentiated thyroid cancer [8, 9, 10, 11, 12, 13, 14, 15, 16] since this technique offers superior spatial resolution compared with SPET.

The aim of this study was to further characterise the mechanisms governing FDG accumulation in thyroid cells. In particular, we sought to investigate the effect of TSH on this variable since evidence from clinical studies suggests that TSH exerts an important influence on FDG uptake in thyroid tissue. We chose primary cell cultures of human thyroid tissue as an in vitro model as primary cell cultures are well established for this purpose and may more closely reflect the in vivo situation than permanent thyroid cell lines [17].

Materials and methods

Thyroid cells. As described in more detail previously [17], human primary thyroid cells were obtained using the following protocol: Samples of thyroid tissue were obtained from three patients undergoing surgical treatment for nodular goitre. Studies were performed on paranodular tissue components exclusively. Specimens were stored in 50-ml tubes in phosphate-buffered saline during transportation. All preparations were performed under sterile conditions in a laminar airflow bench. Tissue components were washed, and connective tissue and nodular goitre structures were separated from macroscopically regular tissue components and discarded. Tissue samples were cut into small pieces using fine surgical scissors and scalpels. Fragments were collected in a tube containing grade II neutral protease (Dispase II, 5 ml/g tissue, >2.4 U/ml; Roche diagnostics Corp., Indianapolis, IN). Enzymatic digestion was maintained over 30 min, finally yielding isolated cells. Isolated thyroid cells were separated by different washing and centrifugation procedures removing connective tissue and supernatant Dispase II.

The resulting cell pellet was resuspended by 10 ml of a culture medium based on Ham F12 solution (Gibco, Karlsruhe, Germany) supplemented by five hormones or growth factors [10 mg/l insulin (Gibco), 5 mg/l transferrin (Gibco), 10 µg/l somatostatin (Sigma, Deisenhofen, Germany), 3,625 µg/l hydrocortisone and 10 µg/l Gly-His-Lys (Sigma)]. In some cases, cells were additionally incubated with bovine TSH (Sigma) in the first period of culture after isolation to stimulate confluent monolayer growing. All media were enriched by 1% heat-inactivated fetal calf serum (Gibco) and a solution of antibiotics (5 ml/l streptomycin/penicillin; Gibco). Approximately 200,000 cells were seeded into tissue culture flasks (Greiner, Frickenhausen, Germany). Before starting experimental studies, cells were plated in 35-mm plastic culture dishes to generate a cellular monolayer. Cells were cultured in a humidified incubator in an atmosphere of 5% CO, 95% air (v/v) at 37°C.

Cell viability. Cell viability was determined by trypan blue staining. Viability assays were performed in duplicate in two cultures. Cell viability exceeded 95% in both cases.

Histochemical staining. To differentiate between fibroblasts, connective tissue and epithelial thyroid cells, immunohistochemical staining using an antibody against the epithelial antigen KL1 was performed. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibodies using commercially available staining methods (APAAP-kit, Dako).

FDG uptake experiments. FDG was diluted in PBS. Between 0.8 and 1.5 MBq [^{18}F]FDG was added to each culture well containing 1.2 ml PBS and incubation continued at 37°C for 1 h if not otherwise indicated. After incubation, the medium was removed and an aliquot was used for radioactivity measurements. Subsequently, the cells were washed twice with PBS. Cells were dissolved from the wells mechanically and the resulting solution was transferred to tubes with a PBS rinse. After homogenisation, an aliquot was taken for radioactivity measurement (Caprac Counter, Capintec) and for protein determination by the method of Bradford [18]. [^{18}F]FDG activity was quantified as percent of whole FDG activity (% ID) or as % ID divided by total protein mass. Cellular washout of FDG and metabolites was determined at time intervals between 1 and 3 h by measuring the radioactivity concentration in the medium after washing with PBS and replacement by FDG-free PBS (1.2 ml/well).

Further incubation studies were directed at investigating the following parameters:

- Incubation time, varied between 15 min and 3 h
- Glucose concentration, varied between 0 and 1,000 mg/dl
- Concentration of TSH, varied between 0 and 1,000 mU/l/24 h
- Time of incubation with TSH, varied between 0 and 72 h

Quantification of Tg. Concentrations of Tg in culture media were measured by radioimmunoassay (Dynotest Tg; Brahms, Henningsdorf, Berlin, Germany) in one preparation. The results were obtained as concentration (ng/ml). For quantification of Tg that had been liberated by thyrocytes, the total value was corrected for the amount of Tg in fresh media, which were a fraction of the fetal calf serum. Consecutive measurements yielded accumulation curves of Tg as a function of time over the total incubation period.

Radio-thin layer chromatography. [^{18}F]FDG metabolites in thyroid cells were investigated by radio-thin layer chromatography on cellulose-coated plates (CEL 300, Macherey-Nagel) and ethanol/1 *M* NH_4OAc 3:2 (v/v) as previously described [19]. Chromatograms were measured using the Instant Imager (Canberra Packard), and metabolites were identified by their Rf values.

Statistics. All experiments were performed in triplicate if not otherwise indicated and repeated independently at least three times. Data are expressed as mean±SEM. The significance of differences in means was examined using Student's *t* test for paired data. *P* values <0.05 were considered significant.

Results

More than 98% of the cells obtained using the above-described procedure expressed the surface marker Kl-1 (Fig. 1).

The concentration of Tg within the incubation media increased significantly with time and approximately doubled at a TSH concentration of 50 µU/ml compared with TSH-free incubation (Fig. 2). There was no significant washout of FDG or FDG metabolites within 3 h. FDG accumulated in the thyroid cells linearly with time, doubling roughly every 20 min (Fig. 3). Uptake was competitively inhibited by unlabelled glucose, being approximately 70% at 100 mg/dl glucose compared with the value measured in glucose-free medium (Fig. 4).

TSH significantly increased FDG uptake in vitro in a time- and concentration-dependent manner: cells cul-

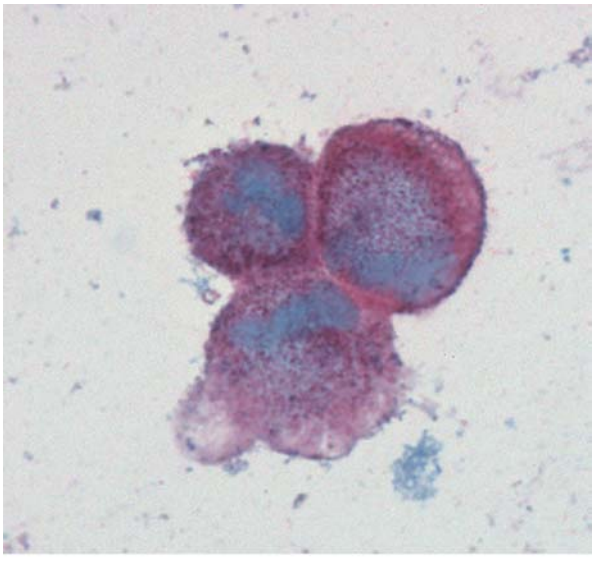

Fig. 1. Immunohistochemical staining of human thyrocytes. Primary thyroid cells were isolated, purified and stained as described in Materials and methods. Cells were maintained in non-coated plastic dishes and stimulated using media containing five different growth factors. More than 98% of cells expressed the cell surface marker Kl-1, specific for cells of epithelial origin

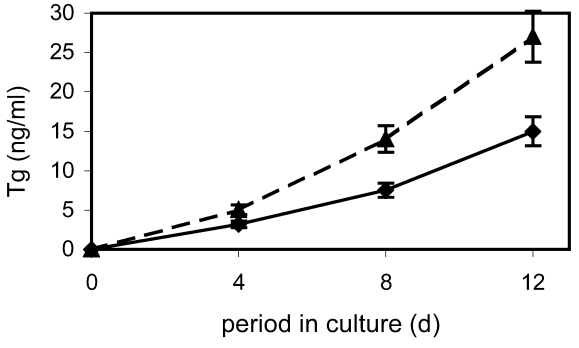

Fig. 2. Tg release by human thyrocytes: 50 µU/ml TSH stimulates Tg synthesis (*broken line*). Values are mean±SEM of one typical experiment performed in triplicate

tured at a TSH concentration of 50 µU/ml doubled FDG uptake (Fig. 5). A significant relationship between FDG accumulation and pre-incubation time was observed, the uptake at 72 h of incubation being approximately 300% of that without TSH pre-incubation (Fig. 6).

FDG-6-phosphate was detected intracellularly using radio-thin layer chromatography (Fig. 7). A second radioactive metabolite representing [^{18}F]FDG-1,6-diphosphate was also detected. The distribution of radioactive intracellular compounds was determined to be the following: FDG-1,6-diphosphate/FDG-6-phosphate/FDG =26:60:14 (% sum of regions). Both radioactive metabolites could be hydrolysed to [^{18}F]FDG by the use of perchloric acid ($HClO_4$).

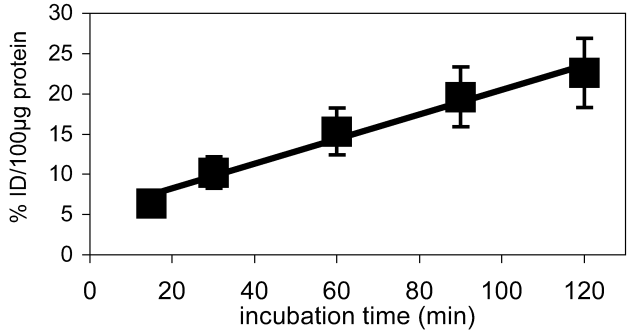

Fig. 3. Time course of FDG accumulation in human thyrocytes. Thyrocytes were incubated for 14 days as described in Materials and methods to generate confluent monolayer. FDG uptake is expressed as % ID/100 µg protein. Data are mean±SEM of four independent determinations within a single experiment. Two similar experiments yielded qualitatively identical results

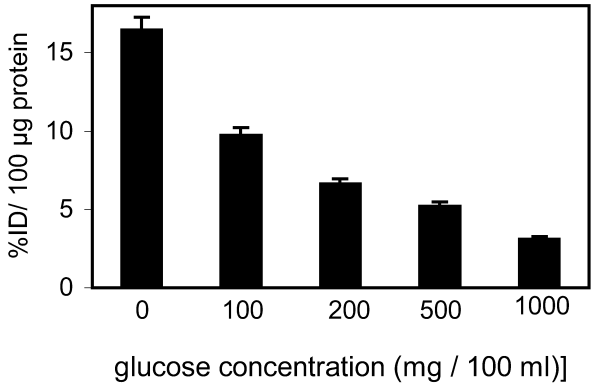

Fig. 4. Primary thyrocytes were incubated for 1 h with FDG as described in Materials and methods. Glucose was added in varying concentrations to simulate hyperglycaemic or hypoglycaemic conditions. FDG uptake decreased with increasing glucose concentrations. Values are mean±SEM of one typical experiment. Two similar experiments yielded qualitatively identical results

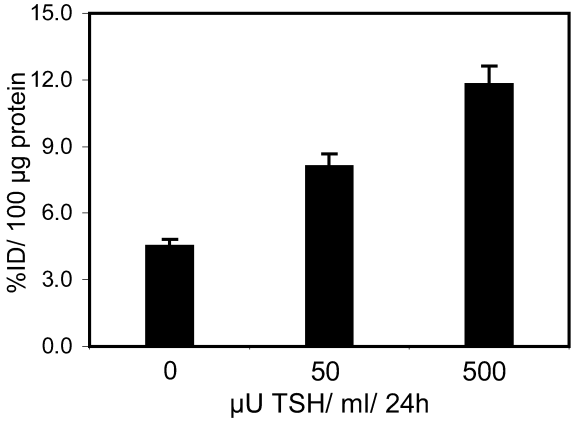

Fig. 5. FDG uptake in human primary thyrocytes. Cells were preincubated with TSH in indicated concentrations for 24 h. Incubation with FDG was performed for 1 h. FDG uptake is expressed as % ID/100 µg total cell protein. Differences were statistically significant (P<0.003 and P<0.002)

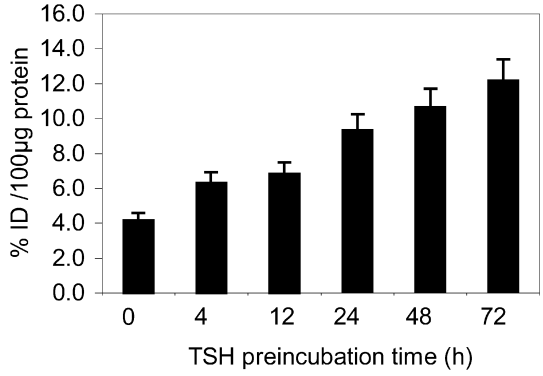

Fig. 6. FDG uptake in human primary thyrocytes. Pre-incubation with 100 µU/ml TSH was performed for the indicated period. Incubation with FDG was performed for 1 h. FDG uptake is expressed as % ID/100 µg total cell protein. Note that the abscissa is not linear

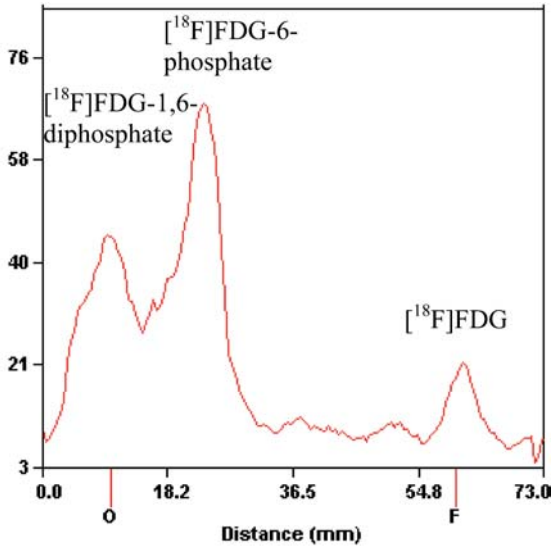

Fig. 7. Radio-thin layer chromatography of intracellular metabolites of FDG in primary thyroid cell cultures. Incubation with [^{18}F]FDG was performed for 1 h as described in Materials and methods. Radio-thin layer chromatography of cellular lysates shows two radioactive intracellular metabolites (*red line*). Both radioactive intracellular metabolites were identified by Rf values and can be hydrolysed to [^{18}F]FDG using $HClO_4$

Discussion

In this study we investigated the uptake of the PET tracer FDG into primary thyroid cell cultures. Primary cell cultures are a well-established model of the functioning, TSH-sensitive human thyroid, though there are well-known limitations inherent to transferring results obtained in vitro to in vivo conditions [20].

Because Tg is a specific product of differentiated thyrocytes, the accumulation of Tg observed in the culture media of our preparations proved that functionally active thyrocytes were present within the potentially heterogeneous cell populations of the primary cell cultures. Using immunohistochemistry, we could further establish that more than 98% of the cells in our preparation were of epithelial origin, thus excluding significant contamination by fibroblasts or other cells from connective tissue and extending our previously published protocol [17].

FDG uptake by primary thyroid cell cultures was competitively inhibited by increasing the glucose concentration in the medium, suggesting that FDG accumulation occurs via specific interaction with transport proteins and enzymes. Furthermore, accumulation of FDG in these cells was linearly dependent on the time of incubation and a consequence of FDG phosphorylation. Therefore, FDG uptake by primary thyroid cell cultures follows mechanisms at least in principle similar to those applicable to most other cells of the human body for which data on FDG uptake are available [21].

As, for example, in macrophages [22], thin layer chromatography of our cell lysates disclosed not only FDG-6-phosphate but also FDG-1,6-phosphate as metabolites of FDG. This is attributable to the little known and as yet not systematically studied role of FDG-6-phosphate as a substrate for phosphoglucomutase and is of minor importance for PET imaging, since both phosphorylated metabolites of FDG remain trapped intracellularly.

The principal finding of our study is that FDG uptake by primary thyroid cell cultures of human origin depends significantly on TSH. To the best of our knowledge, FDG uptake into this cellular model has as yet not been studied. However, Filetti et al. demonstrated in 1987 [23] that the cellular uptake of 2-deoxy-D-glucose and of the glucose transport tracer 3-*O*-methyl-D-glucose, both labelled by carbon-14, was significantly enhanced by TSH in the rat thyroid cell line FRTL-5. They could further show that, in particular, glucose transport across the cell membrane was increased and that this was due to an increased translocation of Glut-1 towards the FRTL-5 cell surface. Their findings may serve as an explanation for our data, though direct confirmation in cells of human origin is awaited.

Further factors governing glucose transport into thyroid cells of animal origin are insulin and iodine [24, 25, 26]. Our media were iodine-free, but contained insulin. Since the concentration of insulin was constant in all experiments, this could not have interfered with our results. Further studies are necessary to systematically study the interrelationship of the effects of TSH, insulin and iodine on the glucose metabolism of thyroid cells.

Since we report FDG uptake in primary cell cultures obtained from benign thyroid tissue only, our findings may not hold true for thyroid cancer. TSH exerts its effect on the thyroid gland mainly via the TSH receptor–Gs protein–adenylate cyclase cascade and—probably to a lesser degree—also via the TSH receptor–Gq protein–phospholipase cascade [27, 28].

It has been shown that the expression level of the TSH receptor messenger RNA in thyroid tumours is related to the level of differentiation and that poorly differentiated thyroid carcinomas may lack TSH receptors completely [29, 30]. Furthermore, even when expressing the TSH receptor, cancerous tissue may react differently to TSH stimulation than normal tissue since somatic mutations of the TSH receptor gene and also other alterations of the cAMP cascade have been detected in thyroid tumours [31, 32]. Therefore, not all thyroid carcinomas would be expected to increase FDG accumulation on TSH stimulation to the same degree as normal thyroid tissue. On the other hand, the majority of differentiated thyroid carcinomas retain TSH sensitivity, as evidenced by their ability to increase iodine uptake and Tg release in hypothyroidism. Thus, although our data do not directly prove the TSH dependency of FDG uptake in thyroid tumours, this relationship seems highly probable in the majority of well-differentiated, still TSH-sensitive thyroid carcinomas. Clearly, an investigation into the signalling pathways linking TSH receptor stimulation to the mechanisms governing glucose transport and metabolism would be interesting in this context.

It is generally accepted that endogenous stimulation of TSH induced by withdrawal of hormone replacement has to precede radioiodine scintigraphy. The cessation of thyroid hormones, however, causes considerable discomfort to patients. The alternative, i.e. the administration of recombinant human TSH, circumvents this problem, but adds further expenses to patient management. Therefore, the ideal diagnostic test in the follow-up of patients with differentiated thyroid cancer would reach high accuracy at low levels of TSH.

Data on the effect of TSH on thyroid uptake of ^{99m}Tc-sestamibi and -tetrofosmin have, to the best of our knowledge, not been published as yet. Using the same cellular model as that used herein we were able to show that the uptake of ^{201}Tl into thyroid cells increases with increasing concentrations of TSH [17]. Therefore, ^{201}Tl-SPET would be expected to be more sensitive at high TSH concentrations, although this assumption still lacks support from in vivo data.

Owing to limitations of the SPET technique, all these radiopharmaceuticals would be expected to have inferior performance compared with FDG. However, several recent patient studies have established a dependence of FDG accumulation on TSH in recurrences of thyroid cancer [33, 34, 35]. Our in vitro data nicely confirm these in vivo findings. Taking these two lines of evidence together, the recommendation to perform FDG-PET in patients afflicted by thyroid cancer at high levels of TSH is strongly supported [36]. On the other hand, for reasons discussed above, it should be borne in mind that dedifferentiating thyroid cancer progressively loses TSH dependency, so that this recommendation can only cautiously be extrapolated to this type of neoplasm. Clearly, in vitro studies in cells mimicking this entity would be interesting to help elucidate this issue. These investigations should also take the other above-mentioned factors potentially governing thyroid glucose metabolism into account.

Conclusion

TSH stimulates FDG uptake by benign thyroid cells in a time- and concentration-dependent manner. This supports the clinical evidence that in well-differentiated thyroid carcinomas, most of which are still TSH-sensitive, FDG-PET is more accurate at high levels of TSH.

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