Insulin Receptor Isoforms and Insulin-Like Growth Factor Receptor in Human Follicular Cell Precursors from Papillary Thyroid Cancer and Normal Thyroid

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Context: Factors involved in the biology of normal and cancer stem/precursor cells from the thyroid are unknown. Thyroid cancer cells are responsive to insulin and IGF-I and IGF-II and often overexpress the insulin receptor (IR) and the IGF-I receptor (IGF-IR).

Objective: We investigated the role of IR isoforms (IR-A and IR-B), IGF-IR, and their ligands in thyroid follicular cell precursors both normal and malignant.

Design: We established cultures of follicular cell precursors as thyrospheres from three papillary thyroid cancers and the corresponding nonaffected tissues. The expression of IR, IGF-IR, and their ligands was evaluated by quantitative RT-PCR and, in one case, also by Western blot. The effects of insulin and IGFs on thyrosphere growth and self-renewal were evaluated.

Results: Thyrospheres were characterized by the expression of stem cell markers and low/absent thyroid specific markers. Thyrospheres from normal tissue, but not from cancer tissue, could be induced to differentiate. Both IR isoforms, IGF-IR, IGF-I and IGF-II, were expressed at high levels in thyrospheres and markedly decreased in differentiating cells. IR-A was the predominant isoform in thyrospheres, especially from cancer, while IR-B was predominant in differentiating cells. Cancer thyrosphere growth was stimulated by insulin and IGFs.

Conclusions: Our data suggest that IR isoforms and IGF-IR play a role in the biology of follicular thyroid precursors. Cell differentiation is associated with marked changes in the expression of these receptors and cognate ligands. These data may provide insight for future differentiation therapies in thyroid cancer. **(*J Clin Endocrinol Metab* 96: 766–774, 2011)**

Insulin, the principal regulator of glucose metabolism, has been recently suggested to have a role in cancer development and progression. Although its mitogenic effect in cultured cells has been known for a long time, this effect has often been attributed to cross-talk with the IGF-I receptor (IGF-IR). However, it has now been clearly established that the mitogenic and antiapoptotic effects of insulin may be directly mediated by its own receptor (1, 2). More recently, overwhelming evidence has linked both obesity and type 2 diabetes mellitus to an increased cancer risk and risk of mortality (3–6). Insulin resistance and compensatory hyperinsulinemia characterize both obesity and type 2 diabetes mellitus and are considered major determinants of the increased cancer risk in these patients (7). In particular, insulin resistance is associated with an increased thyroid volume and an increased risk of thyroid nodules and cancer (8–10). These findings have led to the hypothesis that the rising thyroid cancer incidence observed worldwide might be related to the rising occurrence of insulin resistance (11).

At physiological concentrations, insulin effects are mediated via its receptor (IR), which is expressed in two

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2010-1255 Received June 3, 2010. Accepted November 8, 2010.
First Published Online December 1, 2010
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For editorial see page 610

Abbreviations: Ct, Cycle threshold; HR, IR/IGF-IR hybrid; IGF-IR, IGF-I receptor; IR, insulin receptor; PTC, papillary thyroid cancer.

isoforms: IR-A and IR-B (12). IR-A originates by exon 11 skipping (Ex11-) and differs from the IR-B isoform because it lacks 12 amino acids at the carboxy terminus of the α-subunit. Exon 11 skipping is developmentally regulated, and IR-A is predominantly expressed in fetal tissues. This isoform becomes less expressed in adult differentiated tissues, especially in liver, muscle, and fat, classical targets of the metabolic effects of insulin where the IR-B predominates. IR-A, however, is still expressed in adult tissues, especially in tissues that are nonclassical targets of insulin (13). While IR-B is a highly specific receptor for insulin, IR-A is also a high affinity receptor for IGF-II and binds IGF-I as well, although with lower affinity (12–14). An autocrine IGF-II/IR-A loop has been demonstrated to sustain growth and survival in a variety of cancers, including thyroid cancer especially when dedifferentiated (15).

Recently, the finding that most cancers contain malignant stem cells has led to the hypothesis that malignancies may originate from a small subset of nondifferentiated cells that undergo transformation. These cancer stem/progenitor cells have an unlimited ability for self-renewal and continuously regenerate the tumor bulk, being also responsible for metastatic spread and resistance to conventional anticancer therapies (16). Multipotent stem cells from the adult human thyroid have not been isolated yet (17). However, cells with stem-like characteristics have been isolated from human goiters and thyroid cancer cells by culturing them as nonadherent spheres (18–22) and characterized as stem/progenitor cells, similarly to what described in other organs (23–29). Herein, we investigated the role of IR-A, IGF-IR, and their ligands in thyrospheres obtained from human thyroid cancer and from adjacent normal thyroid tissue.

Materials and Methods

Primary cell cultures and cell lines

Monolayer cultures from normal thyroid tissue were established, as previously described (30), from two patients who had undergone surgery for benign thyroid disease (referred as "P1" and "P2"). The papillary thyroid cancer (PTC) cell line TPC-1 and the colon cancer cell line ARO were provided by Drs. A. Fusco and M. Santoro (Naples, Italy). The human neuroepithelioma cell line SK-N-MC, the human breast cancer cell line MDA-MB-231, and the human cervical cancer cell line HeLa were obtained from the American Type Culture Collection (ATCC, Italy).

Establishment of nonadherent spheres from normal and neoplastic thyroid tissue

To isolate thyroid adult follicular cell precursors from thyroid tissue we adopted a floating sphere culture technique based on an enrichment/purification protocol (20, 31). Thyroid specimens were dissociated with collagenase and single cells obtained by sieving cells through a 40-μm strainer. Cells were resuspended in serum-free DMEM/Ham's F-12 (1:1) medium containing B-27 (1:50, GIBCO), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO), and 20 ng/ml basic fibroblast growth factor (bFGF, Invitrogen, Carlsbad, CA) and seeded at 1×10^5 live cells per 75 cm^2 in low-attachment flasks. Under these conditions, only a small number of cells survived and generated nonadherent clusters, termed thyrospheres.

Thyrospheres were established from three different PTC tissues as well as from the contralateral nonaffected tissues. Case 1 was a 67-yr-old diabetic man with a poorly differentiated PTC within a multinodular goiter with a 4-cm dominant nodule of the left lobe with left laterocervical lymphadenopathy and multiple lung metastases. Histological examination indicated a poorly differentiated PTC extending beyond the capsule and with multiple laterocervical lymphnode metastases. Case 2 was a PTC in an otherwise healthy 39 yr-old woman. Histology indicated a 2.6-cm well/moderately differentiated PTC with multiple locoregional metastatic lymphnodes. Case 3 was a 2-cm well-differentiated PTC from a 70-yr-old woman with a long-standing multinodular goiter.

Additional normal thyrospheres were established from the normal thyroid tissue of five patients who had undergone surgery for benign solitary thyroid nodules. These thyrospheres were pooled and provided an independent control. Samples were obtained from consenting patients, as approved by the Ethics Board at our institution.

Differentiation and characterization of cells from thyrospheres

Cells organized in thyrospheres were dissociated with 0.05% trypsin-EDTA solution and seeded in Petri dishes in DMEM/Ham's F-12 containing 10% FBS at a density of 15,000 cells per 60-mm dish. Cells obtained from normal thyrospheres usually attached after 24 h and grew as monolayers. After three days, TSH (5 mUI/liter) was added to cell cultures. Cells were studied at implantation (day 0) and 3, 6, 9, 15, and 21 d after TSH supplementation.

Cells from normal and cancer thyrospheres, as well as cells differentiating from normal thyrospheres were extensively characterized by measuring both stem cell markers and thyroid specific differentiation markers, using the $\Delta\Delta$Ct method of relative quantification in SYBR Green or TaqMan chemistry. Total RNA (2 μg) was reverse transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems) according to the manufacturer's instructions.

Primers

TaqMan and fluorogenic probes were purchased from Applied Biosystems Assays on Demand Gene Expression Product. GAPDH or β-actin were used to normalize for variation in the amount of input cDNA. Primer sequences are indicated in Table 1.

Gene expression analysis for IR and IR isoforms, IGF-IR, and IGFs

IR and IGF-IR expression was measured by quantitative real-time RT-PCR (qRT-PCR) in cells from thyrospheres both at im-

TABLE 1. Primers used for quantitative PCR

Target gene	Primer sequences (5′–3′)
IR-A	FwTTTCGTCCCCAGGCCATC
	RvGCCCGTGAAGTGTCGC
IR-B	FwTCCTCGTTTAGGAAGACGTTTGA
	RvTTCCGAGATGGCCTAGGGT
IGF-IR	FwTGGTGGAGAACGACCATATCC
	RvCGATTAACTGAGAAGAGGAGTTCGA
IR	FwCGTGGAGGATAATTACATCGTGTT
	RvTGGTCGGGCAAACTTTCT
IGF-I	FwTCGCATCTCTTCTATCTGGCCCTGT
	RvGCAGTACATCTCCAGCCTCCTCAGA
IGF-II	FwGACCGCGGCTTCTACTTCAG
	RvAAGAACTTGCCCACGGGGTAT
β-actin	FwGACAGGATGCAGAAGGAGATCACT
	RvTGATCCACATCTGCTGGAACCT
IR isoforms	FwCCAAAGACAGACTCTCAGAT
	RvAACATCGCCAAGGGACCTGC
Oct-4	TaqMan Gene expression Assay Hs03005111_g1
ABCG-2	TaqMan Gene expression Assay Hs01053790_m1
Sox-2	TaqMan Gene expression Assay Hs0041576_m1
Nano-g	FwCCATCCTTGCAAATGTCTTCTG
	RvCTTTGGGACTGGTGGAAGAATC
CD-133	TaqMan Gene expression Assay Hs01009250_m1
CD-44	TaqMan Gene expression Assay Hs01075861_m1
Tg	TaqMan Gene expression Assay Hs00174974_m1
TPO	TaqMan Gene expression Assay Hs00174927_m1
TSH-R	TaqMan Gene expression Assay Hs01053846_m1
TTF-1	TaqMan Gene expression Assay Hs00201121_m1
Pax-8	TaqMan Gene expression Assay Hs00247586_m1
GAPDH	4310884E Applied Biosystem

Fw, forward; Rv, reverse.

plantation (day 0) and during differentiation, 3, 6, 9, 15, and 21 d after TSH supplementation. Thyroid monolayer cultures were also evaluated. qRT-PCR reactions were performed in an ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) and processed using the absolute quantification assay in SYBR Green chemistry. To test the efficiency of each primer pair, and to generate amplification standard curves of Cycle threshold (Ct) value *vs.* Log copy number, serial dilutions (ranging from 1×10^8 to 1×10^1 copies per ml) of separate plasmid DNAs encoding the genes of interest were used as templates. The pBABE-IRB plasmid was provided by C.R. Kahn (Joslin Diabetes Center, Boston, MA) and the pECE-IGF-IR plasmid was provided by R.A. Roth (Stanford University, Stanford, CA). Comparison of the Ct observed with a specific primer in tested samples with the linear portion of the gene standard curve allowed an estimation of the copy number for each gene, which was normalized to β-actin expression. The copy number of each plasmid was calculated using the following formula: [DNA concentration (g/ml)/plasmid length (bp) $\times$ 600] $\times$ 6,022 $\times$ 10^{23} = molecules/μl. Primer efficiency was determined by the following equation: $E = [10^{(-1/\text{slope})}] - 1$; where slope is the slope of the standard curve when Ct value is plotted *vs.* a known amount of template.

IR isoform expression was measured by RT-PCR analysis using primers for the flanking exons 10 and 12 and resolved on 8% polyacrilamide gel. The 167-bp and 131-bp DNA fragments, representing Ex11+ (IR-B isoform) and Ex11− (IR-A isoform), respectively, were quantified by densitometry on silver stained gel.

The ΔΔCt method of relative quantification and SYBR Green chemistry were used to measure IGF-I and IGF-II mRNA. β-actin was used as an endogenous control for normalization.

Western blotting analysis

Cells were solubilized and subjected to Western blot analysis, as previously described (32), using antibodies against IR (sc-711), IGF-IR (sc-713), and α-tubulin (sc-9104) (Santa Cruz). All immunoblots were revealed by the ECL method (Amersham, Little Chalfont, UK), autoradiographed, and subjected to densitometric analysis using GelEval 1.22 software.

Self-renewal and growth of cancer thyrospheres in response to insulin, IGF-I, and IGF-II

Self-renewal

Cancer thyrospheres, enzymatically dissociated and replated at clonogenic density (25,000 cells per 25 cm^2 low attachment flask) in B27-free medium, were cultured in the presence or absence of either insulin, IGF-I or IGF-II (10 nM) and the IR/IGF-IR inhibitor NVP-ADW742 (1 mM) (Aurogene, Italy), as indicated, to form secondary thyrospheres, which were counted microscopically under a "quadrant grid" at d 8 and 15.

Growth

The volume of secondary thyrospheres was evaluated using an Olympus optical microscope with a DP20-5E digital camera after 15 d treatment with either insulin or IGF-I or IGF-II (10 nM) and NVP-ADW742 (1 mM), when indicated.

Thyrosphere volume (V) was calculated using the following formula: $V = \pi/12 \times ab \times (a + b)$, where ($a$) and ($b$) are the minor and major diameters of each sphere, respectively.

Statistical analysis

Differences between means were analyzed by the Student's t test for unpaired samples. A P value <0.05 was considered statistically significant. Statistical analysis was carried out with Prism 5 software (GraphPad, Software Inc., La Jolla, CA).

Results

Establishment of thyrospheres from normal and thyroid cancer cells

Cells obtained from cancer and normal thyroid tissues were cultured in low-attachment flasks, as described in *Materials and Methods*. By seeding 100,000 cells per 75-cm^2 flask, approximately 40 nonadherent spheroids were observed within 15 d when using normal cells, and roughly 100 spheroids when using cancer cells. Spheres from normal and cancer cells were morphologically different (Fig. 1A). When thyrospheres from normal thyroid tissue were plated in regular Petri dishes in medium containing TSH, they attached and grew as monolayers (Fig. 1B). In contrast, under the same procedure, cancer thyrospheres were unable to attach and remained floating in the

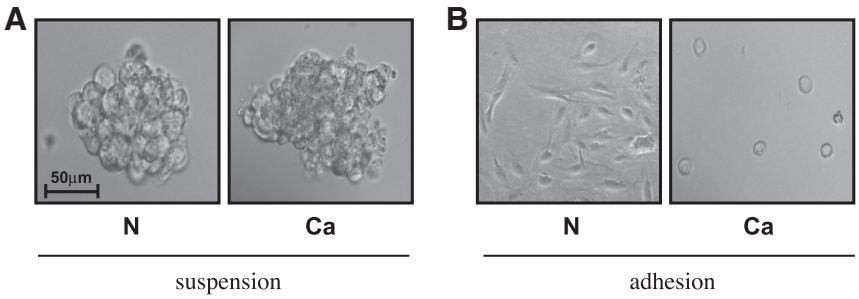

FIG. 1. Morphology of normal and malignant thyrospheres under nonadherent and adherent conditions. A, Representative phase-contrast images of spheres obtained from normal (N) and cancer (Ca) thyroid tissue from case 1 after 15 d of culture in serum-free and anchorage-independent conditions. B, Both normal and malignant thyrospheres were dissociated in single cells and plated in regular Petri dishes in differentiating medium containing TSH (5 mUI/liter) and 10% FBS. Twenty-four hours after plating, cells from normal thyrospheres attached and assumed the morphology of normal thyrocytes growing in monolayers. In contrast, cancer thyrospheres under the same conditions did not attach and remained as single cells in the differentiating medium, even if dishes were coated with type VI collagen. Magnification, ×40.

medium even if dishes were treated with type VI collagen (Fig. 1B). When thyrospheres from both cancer and normal tissue were enzymatically dissociated and replated at clonogenic density they were able to form secondary thyrospheres, showing self-renewal potential (not shown).

Expression of stem cell markers and thyroid differentiation markers in normal and malignant thyrospheres

Stemness markers, including Oct-4, ABCG2, Sox-2, Nano-g, CD-133, and CD-44, were expressed in normal and cancer thyrospheres, but absent in normal thyrosphere-derived adherent cells treated with TSH for 21 d and in primary cell cultures from normal thyroid. HeLa and ARO cells were used as stemness markers positive control (Fig. 2A; see also Supplemental Fig. S1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

In contrast, in both normal and cancer thyrospheres thyroid differentiation markers, including TPO, Tg, and TSH-R, were detected at very low levels (Fig. 2B; Supplemental Fig. S1). Their expression gradually increased in normal sphere-derived differentiating cells with a peak level after 21 d of TSH treatment. ARO cells and normal thyroid cell primary cultures were used as negative and positive controls, respectively. Also TTF-1 and Pax-8 expression was barely detectable in both normal and cancer thyrospheres, as compared with normal thyroid primary cultures (Fig. 2B, Supplemental Fig. S1).

Markers for mesenchymal, hematopoietic, and neural lineage were all negative, showing that no contamination with other cell lineages was present in thyrospheres. When examined by immunofluorescence, the great majority of cells in thyrospheres resulted positive for Oct-4 staining whereas only a very small proportion of cells resulted positive for Tg staining (Supplemental Fig. S1). In contrast, all thyrosphere-derived differentiated cells were strongly Tg positive (Supplemental Fig. S1).

Normal and cancer thyrospheres overexpress IR and IGF-IR as compared with sphere-derived differentiating cells

Expression of IR and IGF-IR was first evaluated in normal and cancer thyrospheres and in normal thyroid primary cultures by qRT-PCR. TPC-1 thyroid cancer cells were used as control. IR expression was very high in both normal (29,000 to 33,000 molecules per μg RNA) and cancer thyrospheres (26,000–65,000 molecules per μg RNA), and it was approximately 30-fold lower in primary cultures from normal thyroid tissue (P1 and P2) and in TPC-1 cells (Fig. 3A). The IGF-IR transcript was highest in normal thyrospheres (99,000–130,000 molecules per μg RNA) and also very high, although more variable, in cancer thyrospheres (49,000–142,000 molecules per μg RNA) (Fig. 3B). The IR:IGF-IR ratio, therefore, was higher in cancer thyrospheres (0.46:0.53) than in normal thyrospheres (0.22:0.31).

In normal thyrosphere-derived cells induced to attach and differentiate (case 1), IR mRNA quickly dropped from 29,000 to approximately 2,000 molecules per μg RNA (d 0–6) (Fig. 3A) and subsequently increased to 3,500 molecules per μg of RNA as the cell differentiation process reached a maximum activity (d 15–21).

IGF-IR mRNA followed a similar pattern: it decreased from 130,000 in thyrospheres to 10,000 molecules per μg of RNA at d 0 and reached a nadir of 7,000 molecules per μg of RNA at d 6. It then increased to more than 20,000 molecules per μg of RNA by d 15–21 in fully differentiated cells (Fig. 3B). Similar IR and IGF-IR mRNA expression patterns were observed in a pool of normal thyrospheres), obtained from five different patients, before and after differentiation in the presence of TSH for 21 d (D21) (Fig. 3, A and B).

In accordance with mRNA overexpression, Western blot analysis indicated that thyrospheres (case 1) had an increased IR and IGF-IR protein content when compared with thyroid primary cultures (Fig. 3C). In differentiated cells, not only receptor expression, but also the IR:IGF-IR ratio decreased as compared with normal thyrospheres (0.17 *vs.* 0.25, respectively).

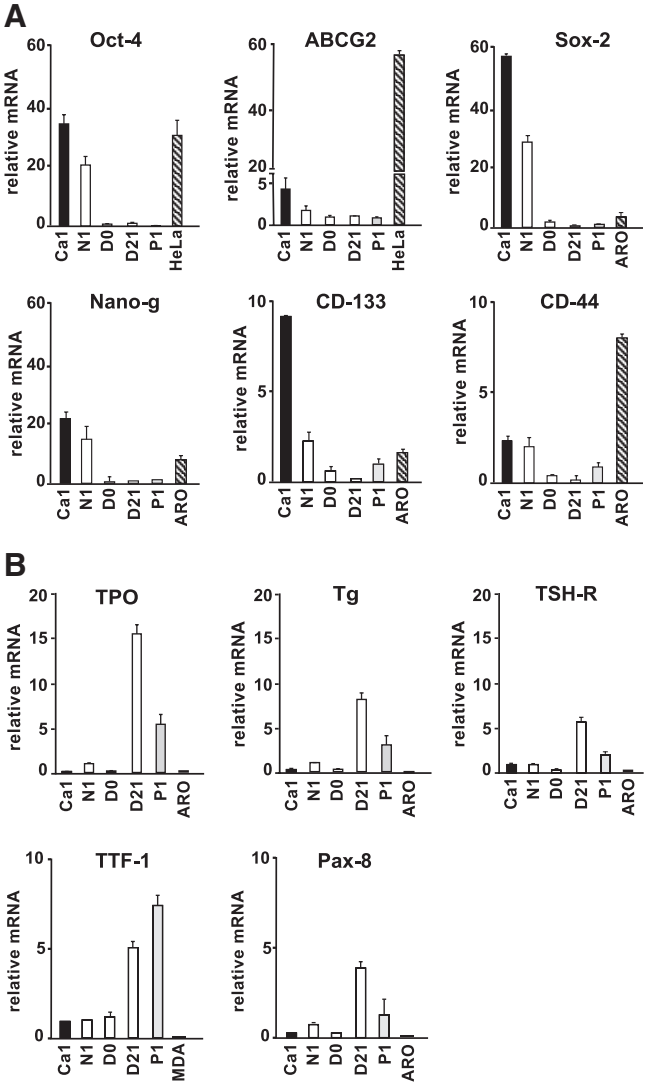

FIG. 2. Analysis of stemness and thyroid differentiation markers. A, Oct-4, ABCG2, Sox-2, Nano-g, CD-133, and CD-44 mRNA expression levels in thyrospheres from cancer (Ca1) or normal tissue (N1) and in monolayer cultures of normal thyrospheres-derived cells at implantation (D0) and at d 21 (D21) in differentiating medium containing TSH (5 mUI/liter) and serum. HeLa and ARO cells were studied as positive control, whereas primary cultures of normal thyroid (P1) treated with TSH (5 mUI/liter) for 7 d were the negative control. B, qRTPCR analysis of TPO, Tg, TSH-R, TTF-1, and Pax-8 relative mRNA levels in the same cell specimens as in A. ARO cells and normal thyroid cells in primary culture (P1) were used as positive and negative controls, respectively. MDA-MB-231 (MDA) breast cancer cells were used as a negative control for TTF-1 expression. Data are presented as the mean ± SE of three separate experiments performed with thyrospheres established from the same donor (case 1). Normalization was done using GAPDH as a housekeeping control gene.

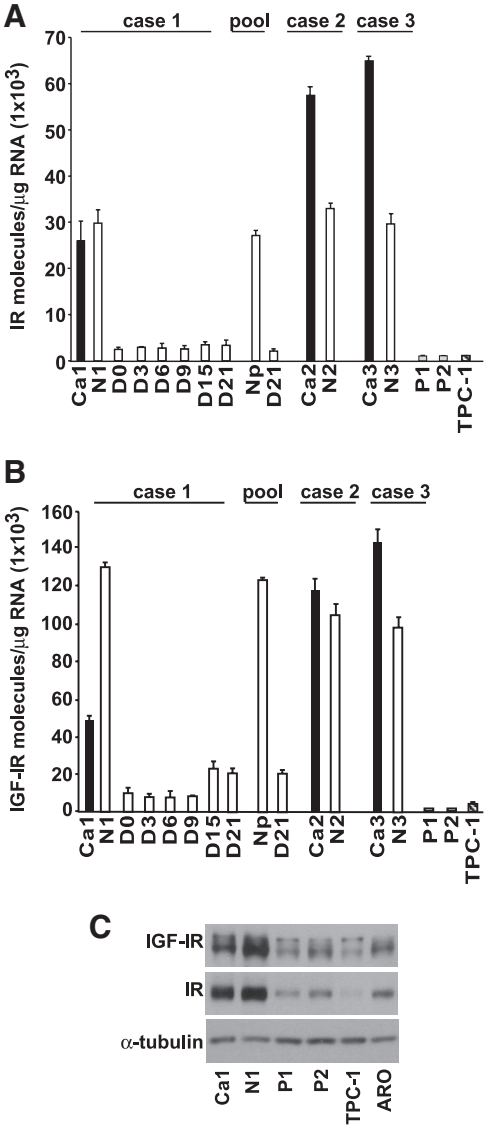

FIG. 3. Expression profile of IR and IGF-IR in normal and cancer thyrospheres. Absolute quantification of IR (A) and IGF-IR (B) expression (molecules per μg RNA) in thyrospheres established from cancer tissues of three different donors (Ca1, Ca2, and Ca3) or contralateral normal thyroid tissues (N1, N2, and N3) and in normal sphere-derived adherent cells from case 1 at implantation (D0) and at d 3, 6, 9, 15, 21 (D3, D6, D9, D15, D21) after exposure to differentiating medium containing TSH (5 mUI/liter) and 10% FBS. TPC-1 thyroid cancer cells and normal thyroid primary cultures (P1 and P2) were studied as controls. A pool of normal thyrospheres, obtained from control patients, was also investigated for IR and IGF-IR mRNA expression before [normal thyrospheres (Np)] and 21 d after (D21) incubation with differentiating medium. C, IR and IGF-IR protein expression. Spheres from cancer (Ca1) and normal thyroid tissue (N1) from case 1 were harvested for protein extraction and analyzed by Western blot analysis. Protein lysates (60 mg) were immunoblotted with the indicated primary antibodies. Normal thyroid primary cultures (P1, P2) and TPC-1 and ARO cells were used as controls.

IR isoform expression in thyrospheres and differentiating cells

Next we evaluated IR isoform expression in thyrospheres. In all cases, the relative abundance of IR-A was higher in cancer thyrospheres (65% to 86%) than in normal thyrospheres (51.5% to 65%) or in thyroid primary cultures (approximately 40%) (Fig. 4A). When normal thyrospheres from case 1 were allowed to differentiate, the IR-A relative abundance decreased from 55% (d 0) to 45% (d 21) (Fig. 4A). Similar results were obtained with pooled normal thyrospheres (not shown).

These data indicate that IR-A is the predominant isoform in thyrospheres and that its level decreases with differentiation.

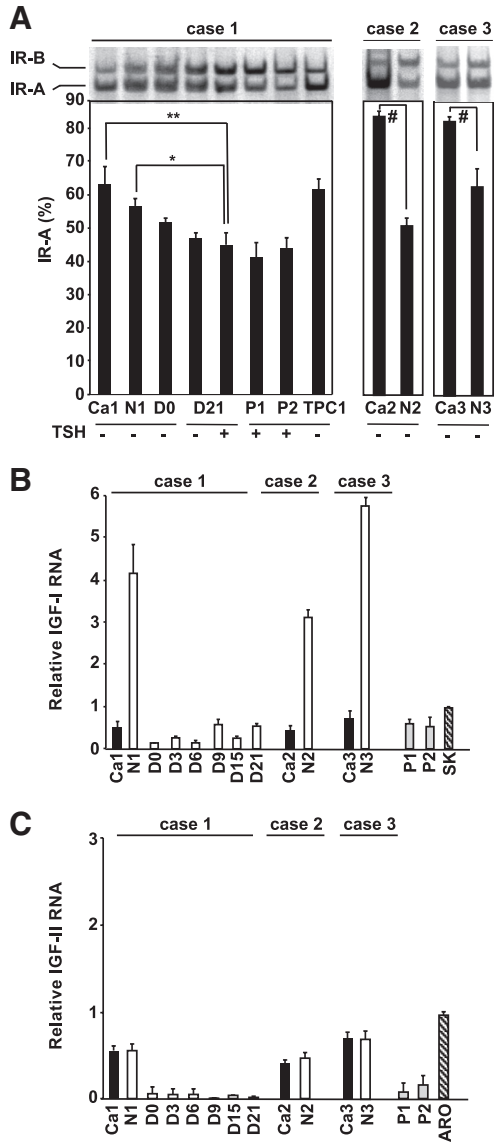

FIG. 4. mRNA expression levels of IR isoforms, IGF-I, and IGF-II. A, IR isoform (IR-A and IR-B) transcripts in spheres obtained from cancer tissues of three patients (Ca1, Ca2, and Ca3), from the contralateral normal tissues (N1, N2, and N3), and from normal sphere-derived adherent cells from case 1 at implantation (D0) and after exposure to medium with (+) or without (−) TSH (5 mUI/liter) for 21 d. *Top panels*, Products of PCR amplification were resolved and silver stained under processing with 8% polyacrilamide gel under nondenaturating conditions. Images of the PCR product from IR-B (Ex+11) and IR-A (Ex-11) are 167 and 131 bp, respectively. *Bottom panels*, Graphical representation of PCR analysis indicating the percentage of IR-A mRNA. The percentage of IR-A mRNA was calculated as follows: densitometric value of band IR-A / densitometric value of bands IR-A + IR-B. Bands were quantified by scanning densitometry using GelEval 1.22 software. All results are expressed as means ± SE of three independent experiments. *, $P = 0.006$, normal *vs.* cancer thyrospheres from case 1; **, $P = 0.009$, cancer spheres *vs.* normal spheres-derived cells from case 1 after treatment with TSH for 21 d; #, $P<0.005$, normal *vs.* cancer thyrospheres from cases 2 and 3 (Student's *t* test). B and C, Relative quantification in SYBR Green chemistry was used to measure IGF-I (B) and IGF-II (C) mRNAs. The conditions were as in Fig. 3. ARO and SK-N-MC (SK) cell lines and primary cultures of normal thyroid (P1 and P2) were analyzed as controls. β-actin was used as an endogenous control for normalization. *Bars*, mean ± SE of three independent experiments.

IGF-I and IGF-II mRNA levels are high in thyrospheres and decrease with differentiation

To evaluate the expression level of IGF-I and IGF-II we first used as calibrators for IGF-I and IGF-II expression SK-N-MC cells (which express IGF-I mRNA) (33) and ARO cells (which express IGF-II mRNA) (15). IGF-I expression was consistently elevated in normal thyrospheres from all three patients studied but substantially lower in both cancer thyrospheres, in differentiating cells from case 1, and in primary cultures from normal thyroid (Fig. 4B).

IGF-II was noticeably expressed in both normal and cancer thyrospheres but absent or very low in differentiating cells from normal spheres and in primary cultures from normal thyroid (Fig. 4C).

Effects of insulin and IGFs on thyroid spheres size and cell renewal

Sphere size is the expression of progenitor cell proliferation and growth, whereas sphere number upon multiple passages reflects the stem cell self-renewal ability (24). We therefore evaluated the effect of insulin, IGF-I, and IGF-II on size and self-renewal of cancer thyrospheres. Sufficient cell material for this experiment was available only from case 1.

Cancer thyrospheres were exposed to 10 nM of each ligand, and the size and number of spheres were scored after 8 and 15 d. Thyrosphere volume increased two- to threefold after 15 d exposure to each ligand ($P < 0.05$ untreated *vs.* treated group). Treatment with the IR/IGF-IR inhibitor NVP-ADW742 (1 mM) significantly inhibited the unstimulated sphere growth by approximately 50% ($P < 0.05$ untreated *vs.* treated group), suggesting that thyrosphere growth is partially dependent on the constitutive activation of the IR/IGF-IR pathway (Fig. 5, A and B). In contrast, only IGF-II at d 15 significantly increased the thyrosphere number (from 23 ± 2 to 32 ± 3, $P < 0.02$) (Fig. 5C).

Discussion

Although multipotent stem cells from the adult human thyroid have not yet been isolated (17), cells with stem-like properties have been found in the normal thyroid, in goiters, and in thyroid cancers (19–22). These cells represent a small minority (<1%) of the entire thyroid follicular cell population and can be isolated by culturing them as nonadherent spheres. This culture system is now widely used to establish long-term cultures enriched in stem/progenitor cells from various organs (23–29). This method is based on the unique property of stem and progenitor cells to survive in serum-free suspension, to produce spherical

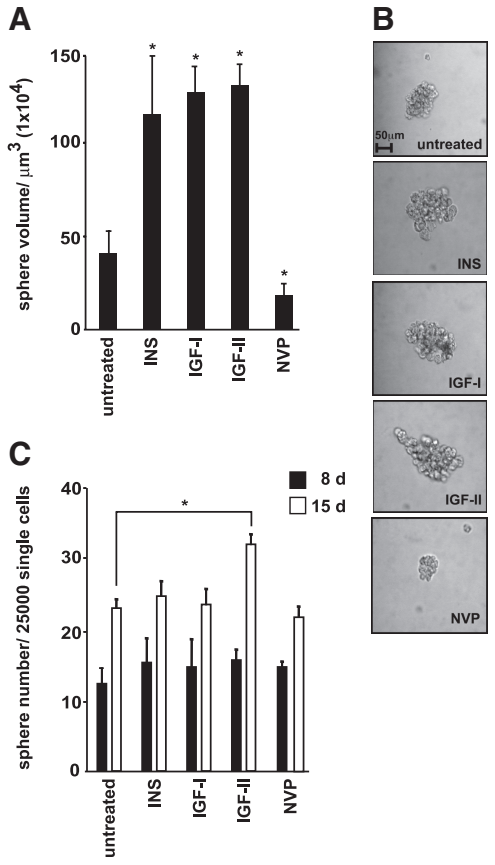

FIG. 5. Size and self-renewal of cancer thyrospheres after exposure to insulin, IGF-I, IGF-II or to an IR/IGF-IR tyrosine kinase inhibitor. A, Cancer thyrosphere growth after exposure to either insulin (INS), IGF-I or IGF-II (10 nM), or NVP-ADW742 (NVP) (1 mM) for 15 d. All three ligands induced a significant increase in sphere volume ($P < 0.05$) when compared with the control group while the IR/IGF-IR inhibitor, NVP-ADW742, induced a volume decrease ($P < 0.05$). *Bars*, mean ± SE of three independent experiments performed with thyrospheres established from the same donor (case 1). Representative pictures are shown in B. Magnification, ×20. C, Cancer thyrospheres were plated at a density of 25,000 cells per 25 cm^2 for 15 d in a nonadherent flask and in the presence of insulin, IGF-I, IGF-II (10 nM), or NVP-ADW742 (1 mM). At d 8 and 15, secondary spheres were counted under the microscope. Three flasks for each condition were analyzed. The results are expressed as a mean ± SE of three independent experiments with thyrospheres established from the same donor (case 1). A statistically significant difference was observed between untreated and IGF-II–treated groups ($P < 0.02$, unpaired Student's *t* test).

colonies that self-renew. Applying this procedure, we obtained thyrospheres with stem/progenitor like cells properties: strongly positive for several putative stem cell markers (Oct-4, ABCG2, Sox-2, CD-133, CD-44, and Nano-g), faintly positive for thyroid specific differentiation markers (Tg, TPO, TSHR), and totally negative for other lineage specific markers (mesenchymal, neural, hematopoietic).

The thyrosphere cells ability to give rise to a second generation of spheres when plated at clonal density provided further evidence for the isolation of stem/progenitor like cells. Furthermore, under differentiating conditions, cells from normal thyrospheres could adhere and differentiate into cells with strong expression of markers typical of well-differentiated thyrocytes (Tg, TPO, TSH-R, TTF-1, Pax-8) and lacking stemness markers. These results are in agreement with the findings of Lan *et al.* (20). Cancer thyrospheres expressed much higher levels of stemness markers than normal thyrospheres, trace levels of TTF-1, Pax-8, and TSH-R and no detectable Tg and TPO transcripts. They also failed to differentiate after exposure to serum and TSH, a finding consistent with the poorly differentiated phenotype of the thyroid cancer studied.

IR and IGF-IR transcripts were markedly higher in both cancer and normal thyrospheres when compared with thyroid primary cultures. Moreover, cancer thyrospheres were characterized by a higher IR:IGF-IR ratio when compared with normal thyrospheres or differentiated thyrocytes. Finally, cancer thyrospheres had a higher IR-A relative abundance (65–86%) when compared with normal thyrospheres (50–65%), differentiated sphere-derived thyrocytes (45%), or normal thyroid primary cultures (40%).

IR and IGF-IR protein content, measured in one case, closely reflected mRNA data.

These findings support the hypothesis that both IGF-IR and IR play a role in thyroid progenitor/stem cells and that thyroid cell differentiation is associated with the down-regulation of IR and IGF-IR and the reduction in the IR-A:IR-B ratio. An inverse relationship has been observed between IR-A levels and differentiation in various cell models (12, 34, 35).

IGF-II is produced at high levels by both normal and cancer thyrospheres likewise by thyroid cancer cells, especially when poorly differentiated (15). It markedly decreases when normal spheres differentiate. Due to the very high content of IGF-IR and IR-A (both high affinity receptors for IGF-II) in normal and cancer thyrospheres, autocrine IGF-II will certainly affect the thyroid progenitor/stem cell biology. The role of IGF-I is probably different: normal thyrospheres express five times more IGF-I and often also more IGF-IR than cancer thyrospheres. Both IGF-I and IGF-IR sharply decrease when thyrospheres are exposed to TSH and then slightly increase at d 21. These data suggest that the IGF-II/IR-A loop is prevalent in cancer while the IGF-I/IGF-IR loop is more important in normal thyroid cells (15, 36). IGFs and insulin significantly stimulated an increase of cancer thyrosphere volume, while treatment with the IR/IGF-IR inhibitor NVP-ADW742 reduced it, providing support to the possibility that the autocrine activation of IR-A and IGF-IR by IGFs has a role in thyrosphere growth. In contrast, it appears that only IGF-II influences cancer thyrosphere self-renewal.

Our observation is limited to three PTC. However, normal thyrospheres included not only the contralateral tissue of those three cases but also a pooled preparation from five different patients. Data obtained were remarkably consistent and suggest that they are representative of a general behavior. Given cancer heterogeneity, however, more work is needed to characterize the involvement of the IGF system in cancer thyrospheres from different histotypes or patient subgroups. These studies will not be easy to perform because most thyroid carcinomas nowadays are diagnosed at early stages and thyrosphere-forming cells are a small minority of the whole cell population.

The scarce availability of cell material prevented further analysis of other IGF system components, such as IR/IGF-IR hybrids (HRs), which are formed in cells containing both IR and IGF-IR by randomly assembly of receptor hemidimers (12, 37). Thus, HRs containing IR-A hemidimers (HR-A, with a higher affinity for IGFs than HR-B) (32) and IR-A/IR-B hybrids (which are functionally similar to IR-A) (12), are expected to be present at a higher level in cancer thyrospheres than in normal thyrospheres or differentiating cells. The role of HRs in thyroid follicular cell precursors is currently unknown.

In conclusion, we found that adult thyroid progenitor/stem-like cells cultured as thyrospheres are characterized by very high IR and IGF-IR content, increased IR-A relative abundance, and high IGF-I and IGF-II expression. All these features decrease in cells undergoing differentiation. Cancer thyrospheres are characterized by increased IR-A relative abundance and IR-A:IGF-IR ratio, compared with normal thyrospheres, and by a relative predominance of IGF-II over IGF-I expression. Both IGFs play a role in cancer thyrosphere expansion while only IGF-II stimulates self-renewal. These data are also intriguing in the context of the reported association between insulin resistance and thyroid cancer (9) and may provide new insights for innovative approaches to thyroid cancer treatment.

Acknowledgments

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This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (to A.B. and R.V.) and from Programmi di ricerca di Rilevante Interesse Nazionale - Ministero Istruzione Università Ricerca Grant 2008BKRFBH_005 (to A.B.).

Disclosure Summary: The authors have nothing to declare.

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