Article title:

Thyroid hormone is a MAPK dependent growth factor for human myeloma cells acting via αvβ3 integrin

Keren Cohen *,†,¶ Martin Ellis *,‡,¶, Shafik Khoury *, Paul J Davis§, Aleck Hercbergs║ and Osnat Ashur-Fabian *,†

* Translational Hemato-Oncology Laboratory, Meir Medical Center, Kfar-Saba, Israel,
†Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
‡Hematology Institute and Blood Bank, Meir Medical Center, Kfar-Saba, Israel
§The Signal Transduction Laboratory, Ordway Research Institute, Albany, NY, USA
║Radiation Oncology, Cleveland Clinic, Cleveland, OH, USA.
¶ These authors contributed equally to this work

Corresponding author: Osnat Ashur-Fabian PhD. Translational Hemato-Oncology Laboratory, Meir Medical Center 59 Tchernichovsky St Kfar-Saba 44281, Israel
Email: osnataf@gmail.com. Phone: +972-9-7472178. Fax: +972-9-7471295

Running title: Thyroid hormones-αvβ3 axis mediates myeloma proliferation

Keywords: myeloma, thyroid hormones, integrins, cancer, MAPK

There are no potential conflicts of interest to disclose

Word count: 5081 words (including abstract)

Total number of figures: 7 figures

Total number of tables: 1 supplemental table
Abstract

Experimental and clinical observations suggest that thyroid hormone (L-thyroxine, T₄, and 3, 5, 3’-triiodo-L-thyronine, T₃) can support cancer cell proliferation. T₃ and T₄ promote both tumor cell division and angiogenesis by activating mitogen-activated protein kinase (MAPK) via binding to a hormone receptor on the αvβ₃ integrin, over-expressed on many cancer cells. We have studied the responsiveness of several MM cell lines to T₃ and T₄ and characterized hormonal effects on cell survival, proliferation and MAPK activation. Overnight T₃ (1-100 nM) and T₄ (100 nM) incubation enhanced, up to 50% (p <0.002), MM cell viability (WST-1 assay) and increased cell proliferation by 30%-60% (p <0.01). Short exposure (10-minutes) to T₃ and T₄ increased MAPK activity by 2.5-3.5-fold (p <0.03). Pharmacological MAPK Inhibition blocked the proliferative action of T₃ and T₄. Antibodies to the integrin αvβ₃ dimer and to αv and β₃ monomers (but not β₁) inhibited MAPK activation and subsequent cell proliferation in response to thyroid hormone, indicating dependence upon this integrin. Moreover, tetraiodothyroacetic acid (tetrac), a non-agonist T₄ analog previously shown to selectively block T₃/T₄ binding to αvβ₃ receptor site, blocked induction of MAPK by the hormones in a dose-dependent manner. This demonstration of the role of thyroid hormones as growth factors for MM cells may offer novel therapeutic approaches.
**Introduction**

Multiple myeloma (MM) remains an incurable disease despite remarkable improvement in survival, particularly in younger patients, in the era of the agents such as thalidomide, lenalidomide and bortezomib. Thalidomide and lenalidomide are immunomodulatory agents with effects on the bone marrow microenvironment rendering marrow less hospitable to myeloma plasma cells as well as inducing apoptosis of plasma cells. Bortezomib is a selective proteasome inhibitor that also exerts its effects in MM by down-regulating the NFκB pathway(1). These novel agents, when used in combination with cytotoxic agents and corticosteroids, achieve overall response rates of 60-90% and median progression free survival of 2-3 years(2). By comparison, treatment regimens consisting only of chemotherapy and corticosteroids that were used in the past resulted in remission rates of 20-35% and progression free survival of 6 to 18 months (3). Current treatment protocols have extended the overall survival of patients with MM, however only 68% of patients less than 45 years of age survive for 5 years from the time of diagnosis, and by 10 years only 55% remain alive (4). Ultimately the disease becomes refractory to all forms of treatment (5) and therefore drugs with new mechanisms of action are urgently needed.

Thyroid hormone has pleiotropic physiological effects on numerous tissues and is essential for normal human development and function. Thus, it is not altogether surprising that the hormone has been shown experimentally to have trophic effects on cancer cells and influence tumor growth and progression (6-8). Indeed, a putative relationship between the thyroid gland and cancer was proposed as early as 1896. A body of epidemiological and clinical evidence has also suggested improved survival in individuals with hypothyroidism who have a variety of tumors (reviewed in (9)). Enhanced response rates to radiation therapy in vitro and in vivo have been reported in hypothyroid cells (10, 11). Conversely hyperthyroidism has sometimes
been shown to be associated with an increased risk of various cancers in large population studies (12-14). An association between thyroid dysfunction and the risk of development of MM has also been demonstrated (15). Such an observation does not mean thyroid hormone is carcinogenic, but does infer the hormone supports tumor cell proliferation.

The cellular mechanisms whereby thyroid hormone simulates cancer cell growth have recently been shown to be, at least in part, due to binding of thyroid hormone to a specific receptor site on the plasma membrane $\alpha_v\beta_3$ integrin (16, 17). The thyroid hormone receptor domain on $\alpha_v\beta_3$ is proximate to the RGD (Arg-Gly-Asp) recognition site (6), through which the integrin interacts with proteins of extracellular matrix (ECM). Via this receptor, thyroid hormone—L-thyroxine ($T_4$) and 3, 5, 3’-triiodo-L-thyronine ($T_3$)—activates the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signal transduction pathways (6). Tetraiodothyroacetic acid (tetrac) and the newly-developed tetrac-nanoparticle (tetrac NP), lack traditional agonist $T_4$ functions and selectively block $T_3/T_4$ binding to the thyroid hormone receptor site on $\alpha_v\beta_3$ (18). These agents reduce cancer cell proliferation, migration and angiogenesis in vitro and in animal models (18-21) and induce apoptosis (22, 23) and DNA double-strand breaks (24). The fact that integrin $\alpha_v\beta_3$ is overexpressed in a variety of cancer cell types (25) and the central role of the MAPK and PI3K cascades in many cancer cells (26, 27), can support concerns that thyroid hormone may promote tumor cell proliferation in the clinical setting (7, 28).

**Materials and Methods**

**Cell lines:** MM cell lines, RPMI 8226, U266 (ATCC; Rockville, MD, USA) and ARP1, ARK and CAG cell lines (established at the Arkansas Cancer Research Center from bone marrow aspirates of patients with MM) were cultured in RPMI 1640 supplemented with 10% heat-
inactivated FBS/antibiotics and were treated with physiological levels of T₄ (100 nM total hormone concentration; 100 pM free hormone) and T₃ (0.1 nM total hormone concentration) as well as supra physiological concentrations.

**Reagents and chemicals:** T₃, T₄, tetrac and RGD/RGE peptides were from Sigma-Aldrich (St. Louis, MO, USA). The MAPK/MEK inhibitor, U0126, was a product of Cell Signaling Technology, Danvers, MA, USA. Monoclonal antibodies against αv (clone P27) and β1 (P5D2) integrins and a β3 (clone H-96) polyclonal antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. αvβ3 (LM609 unconjugated/PE) monoclonal antibody was from Chemicon International, Harrow, UK. MAPK ERK1/2 antibodies were obtained from Cell Signaling Technology and PCNA antibody was a product of Santa Cruz Biotechnology.

**WST-1 cell viability/proliferation assay:** WST-1 (Roche, Mannheim, Germany) (10% final concentration) was incubated with cells at 37°C for 2 h and read using microELISA reader at 440nm.

**Cell counts:** Cells were collected in medium and counted using ADVIA 2120 (Bayer, Tarrytown, NY, USA) cell counter.

**Flow cytometry:** Cell cycle: Cells were harvested, fixed and stained with DNA propidium iodide (PI) (50 μg/mL)/RNAse A (10 μg/mL) (Sigma-Aldrich) and analyzed by FACS. For estimation of αvβ3 abundance in MM cells, cells were harvested in RPMI 1640 and labeled with 10 μg/mL PE-αvβ3 antibody (LM609, Chemicon International) and analyzed by FACS (Navios Flow Cytometer, Beckman Coulter, Inc). Annexin-PI: Cells (10⁵) are incubated with
10 µl FITC conjugated Annexin V / 5 µl PI (MBL, Naka-Ku Ngoya, Japan) and analyzed by
FACS. (Annexin-/PI-, living cells. Annexin+/PI-, early apoptosis. Annexin+/PI+, late
apoptosis).

**Western blotting:** Whole cell lysates were separated on 10-12.5% polyacrylamide gels and
analyzed by western blot using above indicated primary antibodies and appropriate secondary
HRP-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).
Immunoreactive proteins were detected by chemiluminescence reagents (Pierce, Rockford,
IL, USA). Alpha tubulin quantitation normalized the proteins loaded onto the membrane.
Band intensity was visualized and quantified using LAS-3000 (FujiFilm, Japan).

**Immunofluorescence:** Cells were treated, fixed and permeabilized with 0.1% Triton x-100 for
5 minutes at room temperature and then incubated with PE-αvβ3 antibody (LM609,
Chemicon International) or rabbit anti human pERK (Cell Signaling Technology). For
pERK, a secondary FITC goat anti rabbit IgG was used (Jackson ImmunoResearch
Laboratories). Hoechst 33342 was used for nuclear staining (Sigma-Aldrich). Cells were
visualized by a fluorescent microscopy equipped with a camera (Model IX71, Olympus,
Hamburg, Germany) with a 20X/0.50 objective lens and Cell^A (Version 3.1) Olympus
software imaging.

**RNA extraction:** RNA was extracted using NucleoSpine RNA II kit (Macherey-Nagel, Düren,
Germany) according to the manufacturer’s instructions and eluted in 40 µL RNase free water.
RNA concentration and purity were measured using NanoDrop™ 1000 Spectrophotometer
(Thermo Scientific, Wilmington, DE, USA).
cDNA synthesis: 200 ng RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), according to manufacturer instructions.

Real-Time PCR: cDNA was analyzed for apoptotic genes mRNA levels (apaf1, caspase 3, puma, bax, noxa and fas) by 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA), using Applied Biosystems Fast Sybr Green Mix (list of primer sets are depicted in supplementary Table 1). Results, normalized to actin beta, were calculated as fold change using the comparative threshold cycle method (\(2^{-\Delta\Delta CT}\)) relative to control cells (i.e., controls are assigned a value of 1 by definition).

Statistical analysis: Experiments were analyzed using Student's unpaired t-tests for significance (p< 0.05).

Results

Thyroid hormones induce myeloma cell viability and proliferation

The sensitivity of myeloma cells to thyroid hormones was explored by addition of physiological and supra physiological T3 and T4 concentrations (16) to several myeloma cell lines. Two adherent cell lines (CAG and ARK) and two non-adherent cell lines (U266 and ARP-1) were used. In order to adequately control for thyroid hormone concentrations in the experimental setting, cells were grown in the absence of serum for 48 h. T3 was added at modestly supraphysiological and pharmacologic total concentrations (1 nM and 100 nM, respectively) and T4 at physiological and pharmacologic total concentrations (100 nM and 1 \(\mu\)M, respectively). Results (Figure 1A) with physiological and supra physiological thyroid hormone levels indicate a significant increase in cell viability (15-50%). Specifically, T3 at 1
nM increased cell viability by 25% in CAG, U266 and ARK and by 42% in ARP-1 cells. Comparable results were obtained with a higher concentration of T₃ (100 nM) in these cells. Addition of 100nM T₄ resulted in an increase in cell viability of 25% in CAG and ARP-1 cells while a smaller, although significant, effect was seen in U266 and ARK cells. Using supra physiological T4 concentrations induced 20-40% viability in U266, ARP-1 and ARK cells and only a 13% increase in CAG cells. We next evaluated the number of myeloma cells grown in serum-free conditions, followed by treatment with T₃ (1 nM) and T₄ (100 nM). Results (Figure 1B) indicate a significant increase (30%-60%) (p < 0.01) in cell number in T₃- and T₄-treated cells, with the maximal effect observed in ARP-1 cells. Following several experiments in serum-free conditions, CAG cells exhibited the highest tolerability to these condition in comparison to the other cell lines, as demonstrated by a low background of non viable cells (data not shown) and therefore most of the experiments were conducted in CAG cells.

Next, in order to verify the effects of thyroid hormones on myeloma cell proliferation, proliferating cell nuclear antigen (PCNA) protein, a central component in DNA synthesis and replication, was assessed by western blot. In accordance with the increased cell number, a parallel and significant increase in PCNA protein was observed in T₃- and T₄-treated CAG cells in a dose dependent manner (Figure 1C). The effect of T₄ on PCNA protein level was more potent and significant than T₃, with a maximal 2-fold effect at 100 nM T₄ total concentration (p<0.01) which is equivalent to physiologic free hormone concentrations (16). T₃ induced PCNA expression by 20-50% (p<0.01) at supra physiological concentrations only (1 nM-1 μM). Comparable results were obtained in RPMI 8226 cells (data not shown). This increased viability and proliferation was accompanied by a reduced expression of pro-apoptotic genes in CAG cells (Figure 1D). T₄ reduced the expression of apaf1 (26%, p<0.004), caspase 3 (26%, p<0.01), puma (29%, p<0.01) and noxa (28%, p<0.01) while not
affecting the expression of \textit{bax} and \textit{fas}. T3 treatment reduced solely the expression of \textit{apaf1} mRNA by 21% (p<0.002). No significant effect by T3 on the expression of the additional genes examined was observed. Cell cycle analysis revealed an average 30% reduction (p<0.001) in cell death—represented by subG1 cell fraction—in CAG cells exposed to T3 and T4 in serum-free media (\textbf{Figure 1E}). Similar results were obtained in U266, ARK and ARP-1 cell lines (data not shown). \textbf{Figure 1F} depicts a representative cell cycle experiment in which the percentage of subG1 cell fraction (cell death) was reduced from 5.1% (control) to 1.8% and 1.1% in T3- and T4-treated CAG cells, respectively.

\textbf{Thyroid hormones induce MAPK activation in myeloma cells}

We next determined whether the significant induction of viability and proliferation observed in T3- and T4-treated myeloma cells, is MAPK-dependent, similar to results in other tumor types. The results are depicted in \textbf{Figure 2} and show an increase in phosphorylated ERK protein (pERK) in CAG cells treated with T3 (1 nM) and T4 (100 nM). MAPK activation (2.5-fold to 3.5-fold) was observed as early as 10 minutes following T3 (p < 0.003) or T4 (p < 0.03) treatment and was blocked, expectedly, by U0126 (30 \textmu M), a potent MAPK inhibitor (\textbf{Figure 2A}). The activation of MAPK was sustained for up to 24 h (\textbf{Figure 2B}) following T3 (p<0.01) or T4 treatment (p<0.05). T3 increased pERK levels in CAG cells at 1 nM concentration, after which a decline in ERK activity was observed. Activation of MAPK by T4 occurred over a broader concentration range (10-1000 nM). Comparable results were obtained in the RPMI 8226 myeloma cell line (data not shown). This MAPK activation was evident by fluorescent microscopy, using FITC labeled pERK antibody. Microscopy showed pERK protein in the cytoplasm of myeloma CAG cells following treatment with the two thyroid hormone analogues, as indicated by a fluorescent green ring around the blue nucleus (\textbf{Figure 2C}).
Induction of cell proliferation by T3 and T4 is MAPK-dependent

Having shown that T3 and T4 induce both myeloma cell proliferation and MAPK activation, we next examined whether the proliferation observed was MAPK-dependent. Serum starved myeloma cells (CAG, ARK, U266) were treated with U0126 (1μM) MAPK inhibitor at a dose which was shown to partially inhibit MAPK (data not shown) while having a minimal effect on cell viability (Figure 3A-C). After an overnight co-incubation of U0126 with T3 (1 nM) or T4 (100 nM) cell viability and cell cycle analyses were performed. Figure 3A shows that in the presence of U0126, the increase in cell viability induced by T3 was significantly blocked in CAG and U266 cells by 40% and 30% respectively (p<0.02). Similarly, in the same cell lines, the viability induced by T4 was revered by co-treatment with U0126 by 50% and 20% respectively (p<0.04). In ARK cells, a smaller effect (although significant) by U0126 on T3 and T4- induced cell viability was observed. Moreover, cell cycle analysis of CAG, ARK and U266 indicate that U0126 antagonized the lowering effect of T3 on cell death (Figure 3B) by 35%, 48% and 9% respectively (p<0.01, not significant for U266). Similar results were obtained by T4 co-treatment with U0126 in which the reduction in cell death was reversed by 79%, 41% and 15% respectively. (p<0.04). Taken together, the presented experiments indicate that the cell viability produced by thyroid hormone is MAPK-dependent.

αvβ3 abundance in myeloma cells is increased following thyroid hormone treatment and mediates MAPK activation

Since the αvβ3 integrin contains a receptor site for T3 and T4 and we had demonstrated thyroid hormone actions on myeloma cells, we examined the possibility that T3 and T4 can affect the level of αvβ3 integrin abundance. Myeloma cells (CAG, ARK and ARP-1) were incubated with human monoclonal PE-labeled αvβ3 antibody (LM609) and quantified by
FACS for the percentage of $\alpha\nu\beta_3$ positive cells. Results (Figure 4A) show a representative experiment in CAG cells, indicating low levels (0.75%) of $\alpha\nu\beta_3$ abundance in the control cells grown in serum-free (and hormone-free) conditions and an increase to 14.5% and 11%, respectively, after exposure of cells to T₃ and T₄. Comparable results were obtained with ARK and ARP-1 cells (data not shown). The same experiment was evaluated in CAG cells by IF, using human monoclonal PE-labeled $\alpha\nu\beta_3$ antibody (LM609) and nuclear stain. Results revealed a similar increase in $\alpha\nu\beta_3$ abundance in CAG cells, as indicated by the red color around the blue nucleus (Figure 4B). In order to verify identity of the integrin involved in T₃- and T₄-signaling via MAPK, we incubated the cells for 24 h with 10 $\mu$g/mL blocking antibodies to different integrin subunits including $\alpha\nu$, $\beta_3$ and $\beta_1$ monomers before the addition of T₃ or T₄. Results (Figure 4C) show that blocking antibodies to $\alpha\nu$ and $\beta_3$, but not $\beta_1$, completely inhibited MAPK activation by T₃ or T₄. These observations confirm a role for $\alpha\nu$ and $\beta_3$ integrins in T₃- and T₄- signaling in the tested cells. We further examined the effect of functional antibody to the $\alpha\nu\beta_3$ dimer (LM609) on T₃- and T₄-induced MAPK activation. Figure 4D (T₃) and Figure 4E (T₄) show that thyroid hormone-induced MAPK activation was blocked, in a concentration-dependent manner, by pre-incubation with functional anti $\alpha\nu\beta_3$ antibody, supporting involvement of this integrin in hormone action in myeloma cells.

**RGD peptide blocks T₄-induced MAPK activation and enhancement of myeloma cell viability**

The thyroid hormone-binding site on $\alpha\nu\beta_3$ integrin is in close proximity to the RGD recognition site (6, 16) and we therefore determined whether increasing concentrations of RGD peptide added to CAG cells 16 hours prior to T₃ or T₄, would block the hormone-
induced activation of MAPK. The presence of RGD peptide blocked T₄-induced MAPK activation (Figure 5A, lower panel), but did not affect activation of MAPK by T₃ (Figure 5A, upper panel). This differential effect of RGD on the T₄-binding domain is consistent with the subspecialization of the thyroid hormone receptor site on the integrin that has been previously described (7). As expected, control RGE peptide did not block T₄ and T₃ induced MAPK (Figure 5B). Analysis of cell viability in the same experiment showed that RGD treatment significantly inhibited T₄-induced cell viability (Figure 6A), while no effect of RGD on T₃-induced cell viability was observed (Figure 6B).

**Tetrac blocks both T₃ and T₄-induced MAPK activation**

Since tetrac, a T₄ analog with no agonist activity, was shown to selectively block T₃/T₄ binding site upon the αvβ3 integrin in various in vitro and in vivo cancer models(18), we further examined whether tetrac will act in MM cells in a similar manner. Serum starved CAG cells were treated with increasing concentrations of tetrac for 30 minutes before the addition of T₃ (1 nM) or T₄ (100 nM) for an hour incubation. Western results show that while tetrac at 10nM concentration did not block T₃-induced MAPK, at higher concentrations (100 nM-100 μM) ERK phosphorylation was significantly inhibited (Figure 7A). In T₄ treated cells, MAPK induction was blocked when 1 and 100 μM tetrac concentrations were used (Figure 7B). Taken together these results indicate that in MM cells, similar to results in other tumor cell models, tetrac interrupts T₃ and T₄ binding to the receptor site upon the αvβ3 integrin.

**Discussion**

In this paper we demonstrate that thyroid hormone, acting via αvβ3 integrin and MAPK activation, induces cultured human myeloma cell proliferation. Thyroid hormones in
physiological (T₄) or supraphysiological (T₃) concentrations induced myeloma cell proliferation, as evidenced by increased cell viability, cell number and an increase in DNA synthesis. Another mechanism whereby thyroid hormones may increase cell viability can be related to the fact that thyroid hormones, particularly T₄, are anti-apoptotic. This anti-apoptotic activity of thyroid hormone has only recently been reported in several cell and cancer models (29-31). In myeloma cells we have demonstrated a reduction of an array of pro-apoptotic genes, involved mainly in mitochondrial apoptosis.

Our findings are congruent with the recent demonstration that T₃ and, with greater potency, T₄ initiates cell proliferation in a number of tumor cell lines including breast cancer (32), hepatocarcinoma (33), somatotrophic tumor (34), thyroid cancer (29), sarcoma (35) and tumor-associated vascular cells, via activation of a MAPK/ERK1/2 dependent pathway (16). This recently recognized role of the hormone is nongenomic, in that it is initiated at a plasma membrane receptor and does not involve the classical nuclear thyroid hormone receptors (6).

MAPK signaling appears to be critical for the proliferation of myeloma cells. It is frequently upregulated in MM cells (36), significantly contributing to MM cell survival and proliferation, as well as angiogenesis. Our investigation of thyroid hormone-stimulated proliferation in malignant plasma cells revealed rapid activation of MAPK by T₃ at somewhat supraphysiological concentrations and T₄ at physiological and supraphysiological concentrations. Furthermore, the effects of T₃ and T₄ on MAPK activation last up to 24 hours after thyroid hormone treatment. This prolonged effect of thyroid hormones on MAPK activation has also been reported by Davis et al. (17) who observed a secondary increase in MAPK activation at 24 h in glioma C6 cells exposed to thyroid hormone and concluded that this may in fact reflect a secondary increase in an autocrine growth factor such as FGF2. Another example of rapid and sustained MAPK activation is the initiation of angiogenesis...
characterized by a rapid induction of MAPK which is followed by prolonged expression of
the molecule for at least 20 h (37). The proliferative effects of thyroid hormone in myeloma
cells were completely blocked by a specific MAPK inhibitor, U0126, indicating that these
effects are MAPK-dependent. The efficacy of U0126 was consistently higher in CAG cells in
comparison to the other cell lines used, however due to limited data on the status of RAS
mutation and MAPK activity in these cells, this effect is currently under investigation.

MAPK activation by thyroid hormones is independent of the classical nuclear thyroid
hormone receptor as the induction is very rapid (38) and does not require nuclear hormone
receptor, as demonstrated by induction of MAPK activity in HeLa and CV-1 cells that lack
the classical nuclear thyroid hormone receptors (38). Such observations generated the
hypothesis that this thyroid hormone-induced MAPK activity may be initiated outside the cell
nucleus by a non-classical thyroid hormone receptor. Davis et al. established that the
activation of MAPK by thyroid hormone is initiated by binding of T3 and, with a higher
affinity, T4, to the αvβ3 integrin (16) located on the plasma membrane of dividing blood
vessel and tumor cells. This thyroid hormone signal transduction pathway has been reported
in endothelial cells (19), fibroblasts (16) and platelets (39), as well as in a chick
chorioallantoic membrane (CAM) model of angiogenesis (40). The same pathway has been
documented in a variety of cancer cells (6, 7, 17) but have never been studied in
hematological malignancies, such as myeloma.

In the current study, we demonstrate that T3 and T4 up-regulate the cell surface abundance of
αvβ3 on myeloma cell lines. This may be of importance because previous studies of αvβ3
integrin in multiple myeloma have emphasized its role in tumor proliferation and
aggressiveness. Interestingly, αvβ3 integrin has been shown to be involved in myeloma cell
adherence to vitronectin (VN) and fibronectin (FN) (41). It was further shown that plasma cells interacting with VN and FN via the αvβ3 integrin recruit MAPK/ERK1/2 and enhance proliferation and matrix metalloproteinase (MMP)-2 and MMP-9, and urokinase type plasminogen activator (uPA) secretion (42). A recent report also emphasized a role for the αvβ3 integrin in myeloma-related bone disease that is MAPK-dependent (43). We show here that the αvβ3 integrin is involved in T3- and T4-induced MAPK activation and enhancement of proliferation in cultured myeloma cells.

The αvβ3 integrin binds to several ligands. Many integrins, including αvβ3, recognize the ligand tripeptide Arg–Gly–Asp (RGD) (25), through which they interact with proteins of the ECM. Because the thyroid hormone receptor αvβ3 integrin is in close proximity to the RGD recognition site (6), the RGD peptide may be tested in the experimental setting as a probe for participation of the hormone receptor in cellular actions of thyroid hormone. However, misimpressions may be obtained from the use of RGD peptides as probes because the peptides alter inconsistently or only selectively inhibit actions of thyroid hormone that begin at the cell surface receptor (7). We show here that RGD peptide inhibited actions of T4 in myeloma cells, but not the action of T3. These results are consistent with the subspecialization of the thyroid hormone receptor on the integrin which contains distinctive binding sites for T3 and T4 (6, 16). We also studied the action of tetrac in this work. Tetrac is a deaminated derivative of T4 which was previously shown to selectively inhibit the binding of thyroid hormones to the receptor on integrin αvβ3(18). This effect of tetrac was examined in several models of human tumors including glioma(7, 17, 31), thyroid cancer (29), neuroblastoma, osteosarcoma, breast cancer(20) and renal cell carcinoma (21), but was never studied in myeloma. The fact that in this work tetrac effectively blocked induction of MAPK by T3 and
T₄ in myeloma cell model indicates that this action by the hormones was apparently initiated through αvβ3 integrin at the cell surface.

In addition, it is interesting to note that thyroid hormone promotes angiogenesis (44, 45) which has been shown to be of pathogenic importance in myeloma, as demonstrated by the antiangiogenic effects of the effective anti-myeloma therapies thalidomide and lenalidomide (3). Thus tetrac, which inhibits thyroid hormone signaling in myeloma cells, may share these antiangiogenic properties and thus be a useful adjunct to therapy in this disease.

In summary, the present work provides a cellular mechanism whereby thyroid hormones can be considered novel growth factors for myeloma cells via MAPK activation. Since the growth-promoting activity of thyroid hormone is initiated by integrin αvβ3 binding, the effects of antagonizing this molecule in myeloma should be examined.

Acknowledgments

The work of KC was done in partial fulfillment of the requirements for a M.Sc. degree from the Sackler Faculty of Medicine, Tel Aviv University, Israel. The authors wish to thank Hava Avraham, PhD, for helpful comments on the manuscript.
Grant support

This work was supported in part by research grant from the Israeli Myeloma Foundation (AMEN) to OAF and ME.
References:

44. Luidens MK, Mousa SA, Davis FB, Lin HY, Davis PJ. Thyroid hormone and angiogenesis. Vascul Pharmacol. 2010;52:142-5.
Figure legends:

**Figure 1: T<sub>3</sub> and T<sub>4</sub> increase myeloma cells viability.** MM cells (CAG, U266, ARK, ARP-1; 80,000 cell/96-well plate) after an overnight exposure to treatments with T<sub>3</sub> (1 and 100 nM) or T<sub>4</sub> (100 nM and 1 μM) in serum-free conditions were measured for (A) cell viability (WST-1) *, p<0.002 and (B) cell counts, *, p<0.01. Results were repeated 2-5 times, in duplicates and are presented as % of control (average ± SEM). (C) CAG cells were treated overnight with increasing concentrations of T<sub>3</sub> (0.1 nM-1 μM) or T<sub>4</sub> (0.1 nM-1 μM) and analyzed for PCNA protein level (western blots). Results were repeated 3 times, in duplicates and are presented as average ± SEM. *, p<0.01 (D) CAG cells were treated overnight with T<sub>3</sub> (1 nM) or T<sub>4</sub> (100 nM) and mRNA levels of 6 apoptotic genes was analyzed by Real-time PCR. Results were repeated four times, in duplicates and expressed as fold change (2^-ΔΔCT) relative to control cells. *, p<0.01. (E) Serum-free CAG cells were treated overnight with T<sub>4</sub> (100nM) or T<sub>3</sub> (1nM) and assessed for cell death (SubG1 fraction) by cell cycle analysis (Propidium Iodide (PI), FACS). *, p<0.001. Results were repeated 5 times, in duplicates and are presented as % of control (average ± SEM). (F) A representative cell cycle experiment in CAG cells treated with T<sub>3</sub> or T<sub>4</sub>. The SubG1 cell fraction in each treatment is presented. DMSO, used to dissolve the hormones, served as control in all experiments.

**Figure 2: T<sub>3</sub> or T<sub>4</sub> induce MAPK activation in myeloma cells.** (A) Serum starved CAG cells were treated for 10 minutes with T<sub>3</sub> (1 nM, *, p<0.003) or T<sub>4</sub> (100 nM, *, p<0.03) in the presence/absence of overnight incubation with U0126 (30 μM) MAPK inhibitor. pERK and ERK protein levels were measured by western blot analysis. Alpha tubulin was used to normalize the amount of loaded proteins (B) CAG cells were treated overnight with T<sub>3</sub> or T<sub>4</sub> (0.1-1000 nM) after which pERK and ERK were measured by western blot analysis. Experiments were repeated twice and results are presented as average ± SEM. For T<sub>3</sub> and T<sub>4</sub>,
*, p<0.01 and p<0.05, respectively (C) CAG cells were treated with DMSO (left panel), T₃ (1 nM, middle panel) and T₄ (100 nM, right panel) and immunofluorescence (IF) was performed using pERK antibodies (labeled green) and nuclear stain (blue fluorescence). Cells were visualized by a fluorescent microscopy equipped with a camera (Model IX71, Olympus) with a 20X/0.50 objective lens and Cell^A (Version 3.1) Olympus software imaging.

Figure 3: T₃ and T₄ induction of proliferation is MAPK-dependent in myeloma cells.

CAG, ARK and U266 cells were seeded (80,000/96 well-plate) in serum-free media for 48 hours. Cells were co-treated overnight with T₃ (1 nM) and T₄ (100 nM) in the presence or absence of U0126 (1 μM) MAPK inhibitor and measured for (A) cell viability (WST-1; *, p<0.02 for T₃ and p<0.04 for T₄) or (B) cell cycle (PI, FACS; *, p<0.01 for T₃ and p<0.04 T₄). Results, presented as fold from control (DMSO), were repeated for 2-3 times in triplicates.

Figure 4: Integrin αvβ3 is induced by T₃ or T₄ and mediates MAPK activation in myeloma cells. Serum-free CAG cells (250,000/24-well plate) were treated overnight with DMSO, T₃ (1 nM) or T₄ (100 nM), collected and measured for αvβ3 abundance by FACS (A) and IF (B), using phycoerythrin (PE)-conjugated anti αvβ3 antibody (LM609). (C) Serum starved CAG cells were treated overnight with 10μg/ml functional antibodies against the monomer αv (P2W7) or β3 (H-96). β1 integrin antibody (P5D2) served as negative control. T₃ (1 nM) and T₄ (100 nM) were added for 1 hour and pERK and ERK levels were analyzed by western blot. Results were repeated 3 times. Next, serum-free CAG cells were treated overnight with functional antibody against the αvβ3 complex (LM609) and 1 nM T₃ (D) and 100 nM T₄ (E) were added for 1 hour and pERK and ERK levels were analyzed by western blot. Results, repeated twice, are depicted as fold from controls (DMSO). For T₃ and T₄, *, p<0.01 and p<0.002, respectively.
Figure 5: **RGD blocks activation of MAPK by T₄, but not T₃.** Serum starved CAG cells were treated overnight with increasing concentrations of (A) RGD or (B) RGE. Then, T₄ (100 nM) and T₃ (1 nM) were added for 1 hour and analyzed by western blotting for pERK/ERK. Results (average ± SEM) repeated twice are depicted as fold from controls (DMSO). For T₃ and T₄, *, p<0.01 and p<0.02, respectively.

Figure 6: **RGD blocks T₄ but not T₃ induced myeloma cell viability.** CAG cells (grown without serum for 48 hours) were co-treated with increasing concentrations of RGD/RGE together with (A) T₄ (100nM) or (B) T₃ (1nM). 16 hours later, cell viability was examined using WST-1 assay. Results, average±SEM of 2 independent experiments in triplicates, are depicted as fold from controls (DMSO treated cells). *, p<0.04, **, p<0.02.

Figure 7: **Tetrac blocks T₃ and T₄-induced MAPK in myeloma cells.** CAG cells (grown without serum for 48 hours) were pre-treated for 30 minutes with increasing concentrations of tetrac followed by an hour incubation with (A) T₃ (1nM) or (B) T₄ (100nM) and analyzed by western blotting for pERK/ERK. Results (average±SEM) were repeated independently three times and are depicted as fold from controls (DMSO). *, p<0.04, **, p<0.0008.
Figure 2

A. 10 minutes

B. 24 hours

C. Control 1nM T3 100nM T4
Figure 3

A

Viability (% of control)

T3 (1nM)  T4 (100nM)  U0126 (1μM)

-  -  -

-  -  +

-  +  +

B

SubG1 (% of control)

T3 (1nM)  T4 (100nM)  U0126 (1μM)

-  -  -

-  -  +

-  +  +

* indicates significant difference from control.
Figure 4

A

Control

T3

T4

B

0.75%

14.5%

11%

D

T3 (1nM) - + + + -

LM609 (µg/ml) - 0.001 0.01 0.1 0.001

pERK

ERK

42/44kD

E

T4 (100nM) - + + + -

LM609 (µg/ml) - 0.001 0.01 0.1 0.001

pERK

ERK

42/44kD

Graphs showing the percentage of pERK (% of control) with T3 and T4 concentrations, and the effects of LM609 on pERK and ERK levels.

* indicates a significant difference compared to control.
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>T3 (1nM)</th>
<th>RGD</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RGD</td>
<td>-</td>
<td>10nM</td>
<td>1μM</td>
<td>100μM</td>
<td>100μM</td>
<td></td>
</tr>
<tr>
<td>pERK</td>
<td></td>
<td></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>T3 (1nM)</th>
<th>RGE</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RGE</td>
<td>-</td>
<td>10nM</td>
<td>1μM</td>
<td>100μM</td>
<td>100μM</td>
<td></td>
</tr>
<tr>
<td>pERK</td>
<td></td>
<td></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td><img src="42/44kD" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6

![Graphs showing viability (% from control) for different treatments.](image-url)
Figure 7

A

<table>
<thead>
<tr>
<th>T3 (1nM)</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrac (M)</td>
<td>-</td>
<td>$10^{-8}$</td>
<td>$10^{-7}$</td>
<td>$10^{-6}$</td>
<td>$10^{-4}$</td>
</tr>
</tbody>
</table>

pERK

42/44kD

ERK

42/44kD

B

<table>
<thead>
<tr>
<th>T4 (100nM)</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrac (M)</td>
<td>-</td>
<td>$10^{-8}$</td>
<td>$10^{-7}$</td>
<td>$10^{-6}$</td>
<td>$10^{-4}$</td>
</tr>
</tbody>
</table>

pERK

42/44kD

ERK

42/44kD

Graph:

- pERK (% of control)
- Control, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-4}$
- T3, T4
- Statistical significance marked with asterisks: * (p < 0.05), ** (p < 0.01)
Molecular Cancer Research

Thyroid hormone is a MAPK dependent growth factor for human myeloma cells acting via αvβ3 integrin

Keren Cohen, Martin Ellis, Shafik Khoury, et al.

_Mol Cancer Res_ Published OnlineFirst August 5, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-11-0187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mcr.aacrjournals.org/content/suppl/2011/08/05/1541-7786.MCR-11-0187.DC1">http://mcr.aacrjournals.org/content/suppl/2011/08/05/1541-7786.MCR-11-0187.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>