RB-Raf-1 Interaction during Retinoic Acid-induced Differentiation of Human Promyelocytic Leukemia

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Abstract:

All-trans-retinoic acid (RA) is used to treat various types of leukemia through its ability to induce cancer cell differentiation. Methods to enhance the therapeutic use of the anti-cancer properties of RA would further its applications and effectiveness. The purpose of this study is to further analyze the interaction between RB protein, a cell cycle regulator, and Raf-1, a key signaling protein, during RA-induced differentiation of HL-60 cells, bipotent human myeloblastic leukemia cells.

I have already shown that RA promotes this interaction at 24 and 48 hours after treatment, which results in the phosphorylation of RB and progression of the cell cycle, and that an inhibitor of this interaction, RRD-251, enhances the differentiation process by reducing cellular proliferation, increasing the percentage of cells in cell cycle arrest, and increasing expression of markers of differentiation. The particular site of interest on RB is Ser608. Phosphorylation at this site induces a conformational change preventing RB from binding to E2F transcription factors, which facilitate entrance into S phase. Through flow cytometry, I have shown that enrichment of cell cycle arrest by RRD-251 and RA corresponds to unphosphorylated Ser608. It is possible that the phosphorylation of Ser608 is a control mechanism of nuclear Raf-1, as the differentiation process is clearly visible by 72 hours after treatment, when there is no longer an interaction between Raf-1 and RB.

To understand if the dephosphorylation of Ser608 is required for the differentiation process, I plan to create two RB constructs, a phosphomimetic of Ser608 and a phosphodeficient of Ser608 and transfect HL-60 cells with the resulting plasmids. If indeed, the dephosphorylation of Ser608 is required, RA-induced differentiation of cells over-expressing the phosphomimetic should be inhibited, which will be determined by measuring cellular proliferation, cell cycle analysis, and expression of key markers of differentiation. Meanwhile, the phosphodeficient
over-expressing cells will show the consequence of completely preventing the RB-Raf-1 interaction, which should enhance RA-induced differentiation.

Biographical Sketch:

Upon reflection of the first two decades of my life, it is quite surprising that I have turned out to be a scientist. Within my first year of life, my family moved to Torun, Poland, as my mother received a Fulbright Scholarship to teach English. In fact, my first sentence was not in English, but Polish—moje zabawki, meaning my toys, said to my dear older brother. Having returned to the US, my family settled outside of Utica, NY in the house my great-grandfather built. Despite living in Central New York, my international experiences continued as my father is a free-lancer journalist/photographer, having worked for Fox, National Geographic, Smithsonian, and Adventure. When I was 16, I received a State Department scholarship to study intensive Russian, outside of St. Petersburg, Russia, without previously ever speaking the language.

At Cornell, I have continued my study of Russian and have developed a true passion for scientific research. While the combination of Biology and Russian may seem completely unrelated, there is a unifying theme: discovery, whether it is discovery of a new place, culture, or even unknown protein interactions. This desire for exploration/discovery runs deep in my family, from me and my parents, to my great-grandfather who emigrated from Scotland and was an adventurer, dying in the Himalaya in an attempt to discover the fabled Shangri La.

Outside of my studies of Russian and Biology, I am very busy on campus. I joined the Yen lab, where I will be conducting this project, at the beginning of my sophomore year. Since freshman year, I have been a Cornell University Ambassador. Some of my roles have been presenting at information sessions for prospective students, planning events and working at Cornell Days for accepted students, and leading Zinch ambassadors, a way for ambassadors to connect and give more information to prospective students. Last year, I joined a club called Art Beyond Cornell, where I volunteer every week at McCormick Center, a maximum security prison for young men, doing art projects to have the men express themselves and to provide them
with positive role models. This year, I am the Vice President of the club and I coordinate all of
our visits. Lastly, I am involved with Global Medical Brigades, acting as the philanthropy chair.
Last May, I spent a week in rural Honduras on a medical brigade. I am not yet sure about my
future plans at the moment; however I am very committed to continuing my study of Russian,
hopefully through living there, and furthering my scientific pursuits.

Statement of Purpose:

All-trans-retinoic acid (RA) is known to induce myeloid differentiation and cell cycle
arrest in HL-60, bipotent human myeloblastic leukemia cells. This is driven by a novel, durable
signal through MEK-dependent activation of the ERK1/2-MAPK pathway, with the Raf-1
protein as a vital signaling partner (A. Yen, R. Sturgill and S. Varvayanis 2000; Wang 2008;
Hong HY, Varvayanis S and Yen A 2001). Retinoblastoma tumor suppressor protein (RB) is a
key regulator of the cell cycle in HL-60 cells, bipotent human myeloblastic leukemia cells.
Hypophosphorylated RB ostensibly sequesters E2F transcription factors, preventing its activation
HL-60 cells, G1/S/G2 progression is associated with progressively more RB
hyperphosphorylation and hypophosphorylated RB is only detectable after RA treatment (Yen,
Varvayanis 1994). Phosphorylation of RB at Ser608 induces a conformational change that
prevents the binding of RB to the E2F family of transcription factors (Burke JR et al. 2010). RB
is phosphorylated by Raf-1 and previous studies showed that if RB-Raf-1 interaction early in G1
phase is disrupted then RB could not be hyperphosphorylated, even in late G1 phase (Wang S,
could not enter S-phase, which implies that RB phosphorylation by Raf-1 is essential for the
hyperphosphorylation of RB. Thus, if RB phosphorylation by Raf-1 is indeed necessary for cell
cycle progression, then blocking this interaction could be exploited to induce cell cycle arrest.

Kinkade et al. synthesized a small molecule, RRD-251, that selectively inhibited the
binding of Raf-1 to RB (Kinkade R, Dasgupta P, Carie A, et al, 2008). The phosphorylation of

Previously, I found that RRD-251 has a major effect in inhibiting cellular proliferation and inducing cell cycle arrest in HL-60 cells. Additionally, I showed that the treatment of RRD-251 enhanced RA induced differentiation by increasing expression of makers of differentiation, decreasing cellular proliferation, and increasing the percentage of cells in cell cycle arrest. Furthermore, I found that in RA-induced differentiation, RA promoted the phosphorylation of Ser608 by Raf-1 at 48 hours and increased levels of pRBS608 at 24 and 48 hours, but the presence of RRD-251 prevented the interaction and accumulation of pRBS608. Through flow cytometry, I have shown that enrichment of cell cycle arrest by RRD-251 and RA corresponds to unphosphorylated Ser608.

These results indicate the emerging role of RB Ser608 during RA induced differentiation of HL-60 cells. However, it is still not known if the dephosphorylation of pRBS608 is a consequence of differentiation or a prerequisite. Treatment with RRD-251 prevented the phosphorylation of Ser608, which enhanced differentiation by increasing cells in G₀/G₁, increasing expression of key markers of differentiation, and significantly reducing cellular proliferation. Thus, phosphorylation of Ser608, which is induced after RA treatment at 24 and 48 hours, is not necessary to undergo differentiation. When the histograms of pRBS608 are compared to cell cycle analyses, it is clear that enrichment of the lower peak for pRBS608 corresponds to a higher proportion of cells in G₀/G₁. This is expected as phosphorylation of Ser608 induces a conformational change, which prevents RB from binding and sequestering the
E2F transcription factors, thereby preventing its activation of dependent promoters at genes
needed for S phase (Burke JR et al. 2010; Ikeda M., Jakoi L., Nevins J., 1996). However, it is not
yet known if this lower peak corresponds to differentiating cells, as RRD-251 induces a lower
peak for pRBS608 and enrichment of G0/G1, but it does not induce differentiation by itself. This
will be determined by examining samples treated for pRBS608, DNA, and CD11b, a key marker
of differentiation.

The initial interest in Ser608 phosphorylation was to understand the RB-Raf-1 interaction
during RA-induced differentiation. Surprisingly, I previously showed that RA promotes the
nuclear pRBS608-Raf-1 association at 48 hours after treatment and the nuclear accumulation of
pRBS608 at 24 and 48 hours after treatment, but not after 72 hours. These results were consistent
with the previous literature that RA treatment induces the global hypophosphorylation of RB,
only after 72 hours (Yen A, Varvayanis S 1994). Treatment with RRD-251 prevented the
pRBS608-Raf-1 interaction and the nuclear accumulation of pRBS608 at 24 and 48 hours,
thereby enhancing the differentiation process. It is possible that the phosphorylation of Ser608 by
Raf-1 is a regulatory mechanism of nuclear Raf-1, where pRBS608 tightly binds Raf-1
preventing its association with other substrates. The proposed experiment would be to design a
full-length phosphomimetic of Ser608 RB and a phosphodeficient Ser608 RB and to transflect
HL-60 cells. To ensure cells contain the constructs, the plasmid will confer antibiotic resistance,
thus only cells containing the plasmid will grow in culture. Both types of transfect cells will be
treated with retinoic acid and the effect on differentiation will be monitored by cell growth, cell
cycle analysis, and expression of markers of differentiation. Western blotting to determine total
RB expression will be used to check if the cells are over-expressing the plasmid.

Currently, the conjecture is that in cells over-expressing the phosphomimetic of Ser608
the differentiation process should be compromised, while differentiation should be enhanced in
cells with the phosphodeficient Ser608. If Raf-1, a vital signaling protein in the differentiation
process, remains bound to the phosphomimetic, the differentiation process should be inhibited.
Meanwhile, the phosphodeficient RB should not be bound by Raf-1 or phosphorylated at Ser608. This could potentially explain how treatment of RRD-251, which prevents the Raf-1-pRBS608 interaction, enhances differentiation, where RB can sequester E2F transcription factors, preventing entrance into S phase, and where Raf-1 can associate with its various partners to drive the differentiation process. Immunoprecipitates of Raf-1 and its targets should be used to study the effect of the phosphomimetic and phosphodeficient RB on the activity of Raf-1.

Retinoic acid has been shown to induce transient remissions in acute promyelocytic leukemia (APL). Thus, the study of enhancing RA-induced differentiation is critical. Understanding the role of the RB-Raf-1 interaction and RRD-251 warrants further study, as preventing this interaction greatly enhances RA-induced differentiation. These results along with previous studies point to the widespread application of RRD-251, from enhancing RA-induced differentiation of acute myeloid leukemia, to preventing angiogenesis in drug-resistant melanoma, and the subsequently potential therapeutic value of preventing the RB-Raf-1 interaction.

Bibliography:

1. Yen A., Sturgill R., Varvayannis S., Retinoic acid increases amount of phosphorylated RAF; ectopic expression of cFMS reveals that retinoic acid-induced differentiation is more strongly dependent on ERK2 signaling than induced GO arrest is. In Vitro Cellular & Developmental Biology – Animal, 2000, 36, 249-255.

