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An Oct-1-based, feed-forward mechanism of apoptosis inhibited by co-culture with Raji B-cells: Towards a model of the cancer cell/B-cell microenvironment

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Abstract

A continuing conundrum of cancer biology is the dichotomous function of transcription factors that regulate both proliferation and apoptosis, seemingly opposite results. Previous results have indicated that regulated entry into the S-phase of the cell cycle can be anti-apoptotic. Indeed, tumor suppressor genes can be amplified in tumors and certain, slow growing cancers can represent a relatively poor prognosis, both phenomena likely related to reduced cancer cell apoptosis, in turn due to reduced, unproductive entry into S-phase. In this report, we demonstrate that the Oct-1 transcription factor, commonly considered pro-proliferative, indeed facilitates IFN- γ induced apoptosis in 5637 bladder carcinoma cells, consistent with the role of the retinoblastoma protein in down-regulating Oct-1 DNA binding activity and in suppressing IFN- γ induced apoptosis. More importantly, despite the commonly appreciated process of IFN- γ induced apoptosis, IFN- γ at low concentrations stimulated bladder cancer cell proliferation, consistent with apoptosis being dependent on an overstimulation of what is otherwise a pro-proliferative pathway. This observation is in turn consistent with a feed forward mechanism of apoptosis, whereby transcription factors activate proliferation-effector genes at relatively low levels, then apoptosis-effector genes when the transcription factors over-accumulate. Finally, Oct-1 mediated apoptosis is inhibited by co-culture with Raji B-cells, raising the question of whether the normal lymph node environment, or other microenvironments with high concentrations of B-cells, is protective against Oct-1 facilitated apoptosis?

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Conflict of interest statement

No conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yexmp.2014.09.010>.

Keywords

Feed-forward apoptosis; Transcription factors; Oct-1; Interferon-gamma; Bladder carcinoma; B-cell microenvironment

1. Introduction

Most, if not all pro-proliferative transcription factors (TFs) also induce apoptosis (Abell et al., 2005; Field et al., 1996; Hafezi et al., 1999; Lee et al., 2014; Marti et al., 1994; Mauro and Blanck, 2014; Sikora et al., 1993; Yamasaki et al., 1996; Zhao et al., 2000). In fact, one of the oldest and most unexpected results of tumor biology, yet to be satisfactorily explained, is the increased incidence of tumor formation in mice lacking the pro-proliferative transcription factor, E2F-1 (Field et al., 1996; Yamasaki et al., 1996). We have recently proposed a feed-forward mechanism of apoptosis whereby TFs that accumulate to meet the needs of S-phase, for example by activating histone genes, eventually accumulate in such high concentrations that these TFs activate apoptosis genes (Mauro and Blanck, 2014). The requirement of the higher concentration of TFs is based on the observation that apoptosis-effector genes are generally smaller, and have fewer shared-TF binding sites (TFBS), than do the proliferation-effector genes, as well as fewer regions of active chromatin (Mauro and Blanck, 2014). This fact has led to the proposal that, as active TFs accumulate, the TFs first encounter and activate proliferation-effector genes, through what is essentially a stochastic process. If the cell divides and the TF concentration subsides, the process repeats. If S-phase does not proceed normally, the TFs accumulate to such a degree as to populate the apoptosis-effector genes.

In this report, we describe the first empirical representation of this feed-forward process via Oct-1 and via IFN- γ treatment of the 5637 bladder carcinoma cells. Oct-1 has been shown to be de-activated by retinoblastoma protein (Rb) expression, which in turn reduces IFN- γ induced apoptosis in the 5637 bladder carcinoma cells (Fig. 1) (Berry et al., 1996; Osborne et al., 2001; Xu et al., 2009). Furthermore, we determine how this Oct-1 based process is affected by Raji B-cells as a first step in understanding the impact of a B-cell microenvironment on this mechanism of apoptosis.

2. Materials and methods

2.1. Generation of the plot indicating the number of Oct-1 binding site per gene

The assessment of the number of Oct-1 sites per gene, as a function of quality (Z-score) was performed exactly as described (Mauro and Blanck, 2014), except in the previous work TFs were combined in a set whereas for this report only the Oct-1 TFBS was assessed. The Perl (version 5) code for interrogating the human genome database (hg19) is in the supporting online material (SOM) and the Excel file representing the output of the processing for Fig. 2 is also in the SOM.

2.2. Cells and cell culture

All cells were grown in RPMI with penicillin, streptomycin, pyruvate, and 10% fetal bovine serum exactly as described (Palubin et al., 2006). G418-resistant 5637 bladder carcinoma transformants, originally described and characterized in ref. Palubin et al. (2006), were maintained in 400 µg/ml of G418 until one passage prior to use in experiments.

2.3. DAPI chromatin fragmentation assay of apoptosis

Cells were mechanically removed from tissue culture plates, leading to a relatively high level of membrane permeability (Batista et al., 2010). Cells were recovered by centrifugation and resuspended in 1% human serum in PBS at 4 °C, incubated with 0.1 µg/ml DAPI and assayed for fragmented chromatin by flow cytometry (Wen et al., 2001). Flow cytometry analyses were done with a Becton Dickinson LRS II flow cytometer. DAPI was excited with a 405 nm laser and was detected at 450/50 nm, eFluor-670 (eBioscience; see subsection below, Co-culture of Raji B-cells with 5637 bladder carcinoma cell transformants) was excited with a 633 nm laser and detected at 660/20. Tumor cell and B-cell scatter gates were set up on one single cell line samples so that the gates would have minimal contamination from the other cell types, using forward and side scatter measurements and by labeling Raji B-cells with eFluor. Data were analyzed on BD FACS Diva 6.1.3.

2.4. IFN-γ based assay for cell growth

Cells were plated in 96-well tissue culture plates. To obtain an equivalent and useable plating density for two rows, each 96-well plate, with 8 rows, received serial dilutions of cells such that four levels of cell plating densities were available throughout the plate. After plating, each well contained 250 µl of media. IFN-γ was added the following day as a serial dilution across 4 rows and 4 rows were mock treated, except in the case of the C1 cells. 12.5 µl of IFN-γ was added to the first well of every other row. 125 µl of media was removed from each subsequent well and 125 µl of media from the first well was added to the next well, and so on, until 9 wells were treated with increasingly dilute concentrations of IFN-γ. The IFN-γ concentration of the first well was 5000 units/ml and the concentration of IFN-γ in the last well was 19.5 units/ml. All wells were brought to 250 µl total volume following the serial dilution of the IFN-γ. The cells were stained with crystal violet five days following IFN-γ treatment and the staining intensity was quantified with a BioTek FLx800 fluorescence microplate reader.

2.5. Co-culture of Raji B-cells with 5637 bladder carcinoma cell transformants

To perform the co-culture experiments, the 5637 bladder carcinoma cell transformants, AS1, AS3, C1 and C2, from ref. Palubin et al. (2006) were plated at 50% confluent on 60-mm tissue culture dishes on Day 1. A saturated culture of Raji B-cells was split 1/4 the same day for co-culture with the 5637 bladder carcinoma transformants on Day 2. The Raji B-cells were HLA-typed prior to use for verification of cell line identity (SOM). The adherent 5637 bladder carcinoma cell transformants were labeled in the early morning on Day 2 with eFluor 670 (eBioscience) according to the vendor's instructions. Eight hours later on Day 2, media was removed from the tissue culture plates and 2 ml of Raji B-cell culture,

representing approximately 5×10^5 cells, with or without 400 units/ml of IFN- γ (R and D systems) was added to the plates for overnight incubation. The following day, both the supernatant and the adherent cells were collected, centrifuged, and resuspended in 1% human serum in PBS and 0.1 μ g/ml DAPI for flow cytometry.

2.6. Microscopic assay of apoptosis in the presence of Raji B-cells

Cells were plated on 35 mm glass bottom dishes (World Precision Instruments, Inc.) and the co-cultured with Raji B-cells as described in the above methods section. The dishes were examined through a 20 $\times$ /0.5 NA objective lens on an automated Z1 Observer inverted fluorescence microscope with a fully enclosed incubation system to provide an environment of 37 $^{\circ}$ C/5% CO $_2$ (Carl Zeiss GmbH, Germany). Phase contrast images were captured every 30 min for 130 h with an AxioCam MRm3 CCD camera and Axiovision software version 4.8.2 (Carl Zeiss GmbH, Germany). Images acquired were exported as merged and individual channel uncompressed .tif files.

3. Results

We recently observed that in general, proliferation-effector genes are larger than apoptosis-effector genes and have more TFBS for the TFs that are shared by the two gene sets (Mauro and Blanck, 2014). To determine how Oct-1 in particular, was distributed among the proliferation-effector genes and apoptosis-effector genes, we searched the hg19, human genome database, for Oct-1 binding sites among sets of proliferation-effector genes and apoptosis effector genes, obtained by Keyword search of the database (Keyword sets). This process was exactly the same as described in ref. Mauro and Blanck (2014), except that the results were limited to Oct-1. Also as described in ref. Mauro and Blanck (2014), we obtained the Oct-1 TFBS information for A sets and B sets, two additional sets of proliferation-effector and apoptosis-effector genes obtained by inspection of the Keyword sets to eliminate genes that potentially would not be considered strictly members of the category of proliferation- and apoptosis-effector genes or to eliminate multiple members of the same gene families.

Inspection of Fig. 2 reveals that, different from the results obtained with a combined set of TFs that regulate both proliferation- and apoptosis-effector genes, the Oct-1 TFBS is more common in the apoptosis-effector genes in the Keyword set, particularly in the mid-region of the plot, considered to be more relevant than either ends of the plot, due to the construction of the algorithm for establishing the Z-scores. In A and B sets, the Oct-1 binding sites are less numerous in the apoptosis-effector genes. Overall, these analyses indicate that Oct-1 is common to both proliferation- and apoptosis-effector genes.

Oct-1 activity is reduced by the retinoblastoma tumor suppressor protein (Rb), which in turn inhibits IFN- γ induced apoptosis (Fig. 1) (Berry et al., 1996; Osborne et al., 2001; Xu et al., 2009). Thus, we hypothesized that the knockdown of Oct-1 would reduce IFN- γ induced apoptosis, as indicated in Fig. 3 and S1A–H, (SOM), using 400 units/ml of IFN- γ , identical to the concentration used in the previous work demonstrating the inhibition of IFN- γ induced apoptosis by Rb.

Despite the commonly accepted notion that IFN- γ is exclusively proapoptotic in all non-lymphoid settings, we considered the possibility that the albeit disparate roles of Rb and Oct-1 in IFN- γ induced apoptosis indicated that IFN- γ could also stimulate cell proliferation. Thus, we exposed 5637 bladder carcinoma cells transformed with an Oct-1 KD vector, and the control 5637 transformants, with increasing amounts of IFN- γ , with results indicating that in both cases a growth spurt is observed at the lower concentrations and in both cases a decrease in proliferation is observed at the higher IFN- γ concentrations (Fig. 4A, B). Furthermore, the growth spurt was detectable as slightly more rapid for the control transformants containing Oct-1 (Fig. 4C; SOM). Note that the initial cell concentrations for each IFN- γ exposure for Figs. 3 and 4 are different, as detailed in the Materials and methods, and thus the impact of the IFN- γ concentrations can only be compared within the respective experiments.

To determine whether the Oct-1 mediated apoptosis was reduced in the presence of B-cells, we co-cultured the Oct-1 KD transformants, and control transformants, with Raji B-cells and assayed for IFN- γ induced apoptosis using the relatively high concentration of IFN- γ , 400 units/ml, and a DAPI-based, chromatin fragmentation assay (Fig. 5). Results indicated that the B-cells reduced the level of apoptosis in the Oct-1-positive, control transformants. Interestingly, one of the Oct-1 KD transformants, A1 cells, evinced more apoptosis in the presence of B-cells. We suspected that these results were within the limit of experimental variation. Thus, we assayed for apoptosis in the presence of Raji B-cells using another approach, microscopic inspection of the formation of apoptotic bodies and of monolayer destruction. As seen by comparison of Fig. 6C and E, IFN- γ leads to formation of many cells with highly refractive apoptotic bodies and has a highly destructive effect on the A1 cell monolayer, however, the refractive cells are not present, and the destructive effect of IFN- γ on the monolayer is delayed, in the presence of the Raji B-cells.

4. Discussion

Many proliferation-effector and apoptosis-effector genes are regulated by the same transcriptional activators, and despite some dramatic reports supporting this concept, such as the increased number of tumors in mice lacking the pro-proliferative transcription factor E2F-1, there is very little understanding of what distinguishes the function of the shared TFs in distinguishing between these two essentially opposite cell fates. We recently described a potential process whereby accumulation of shared TFs could first lead to activation of proliferation-effector genes and over-accumulation of these TFs could lead to activation of apoptosis-effector genes. This possibility is based on the fact that apoptosis-effector genes are smaller than proliferation-effector genes have fewer shared TFBS (Mauro and Blanck, 2014).

As a test of the above “over-accumulation” hypothesis, we treated cells with increasing amounts of IFN- γ in a setting where IFN- γ induced apoptosis was dependent on one of the TFs that is shared by proliferation-effector and apoptosis-effector genes, namely Oct-1. Results above indicated that small amounts of IFN- γ lead to cell proliferation but that high amounts lead to apoptosis. This is the first report of IFN- γ treatment having opposite effects depending on its concentration, and is reminiscent of a few other signaling pathway

scenarios whereby low-level activation leads to cell proliferation and higher level activation leads to apoptosis, such as activation and hyper-activation of the T-cell receptor, which leads to proliferation or apoptosis, respectively (Carreno et al., 2006; Gatzka and Walsh, 2007). The generality of this phenomenon is to be determined, but based on the “TF-over-accumulation” hypothesis, we expect that all or most pro-proliferative signaling pathways will lead to apoptosis at a high enough signaling level.

Results above also indicated a slight difference in the rapidity of the response to the lower concentrations of IFN- γ , with Oct-1 KD cells having a lower, initial level of cell proliferation. Considering that the Oct-1 KD cells are not likely to be composed of 100% Oct-1 KD cells, due to the loss of the antisense vector, and that the effect of the antisense vector is a knockdown rather than a knockout, and that other TFs are likely to regulate the IFN- γ pro-proliferative response, detecting this slight reduction in proliferation in the Oct-1 KD cells is striking and further con-firms the basic idea: Oct-1 first facilitates replication then apoptosis, as IFN- γ signaling becomes more robust.

Finally, data above are consistent with Raji B-cells inhibiting the IFN- γ induced apoptosis of the bladder carcinoma cells. The apoptosis was not inhibited by normal B-cells from peripheral blood lymphocytes, and though a negative result, this raises the question of whether the Raji B-cells have more of a lymphoblast quality that might be relevant to the inhibition of surrounding tumor cell apoptosis, perhaps by secretion of an anti-apoptotic factor? Alternatively, the Raji B-cells may be interfering with the action of IFN- γ on the tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Summary of the linkage between Rb function and Oct-1 DNA binding activity, based on refs. Osborne et al. (2001, 2004), Pillai et al. (2013), Xu et al. (2009).

Fig. 2.

Verification of Oct-1 binding sites over a range of quality scores (Z-scores) in proliferation-effector genes (lighter squares) and apoptosis-effector genes (darker diamonds). The list of genes used for these analyses is in ref. Mauro and Blanck (2014) and is based on a Keyword search of the human genome annotations (A) as describe in ref. Mauro and Blanck (2014); or is based on inspection of the Keyword set with the removal of all possibly ambiguous genes, termed A sets in ref. Mauro and Blanck (2014). The results related to Oct-1 binding sites for A set genes are in the SOM. The A sets were further reduced by the removal of all duplicate family members, for example, duplicate histone genes or other very closely homologous genes. These final sets were termed the B sets (B). The lists of the genes for all three of these sets are in ref. Mauro and Blanck (2014). The number of the genes for all three sets is at the top of the respective files in the SOM.

Fig. 3.

Cell viability following treatments with 400 units/ml IFN- γ . A1 and A3 represent Oct-1 antisense transformants; C1 and C2 represent G418-resistant control transformants. The isolation and basic characterization of all four transformants is in ref. Palubin et al. (2006). The assay for cell viability is described in Materials and methods and in the SOM.

Fig. 4.

Assay for IFN- γ stimulation of cell proliferation. C2 (A) and A3 (B) cells were plated in 96 well plates and treated with serial dilutions of IFN- γ (A and B) as indicated in Material and methods, or left untreated (B, grey line). After 5 days, the tissue culture wells were stained with crystal violet and quantified. The actual wells used for Fig. 4A are in the SOM. Furthermore, when results from C1, C2, A1 and A3 are all taken into account, it is apparent that the initial growth spurt in response to IFN- γ is greater in the control transformants (C), with a p-value of 0.05 (details in SOM).

Fig. 5.

Cell viability in the presence of IFN- γ and Raji B-cells. A1, A3, C1 and C2 cells were treated with 400 units/ml IFN- γ , or left untreated, in the presence of Raji B-cells and assayed for apoptosis as described in Materials and methods. See also SOM for Fig. 3. HLA typing of the Raji B-cells, to verify cell line identity, is in the SOM.

Fig. 6.

Assay for apoptosis of IFN- γ treated A1 cells in the presence of Raji B-cells, as indicated in the figures (A–H). A1 apoptosis is detected by the formation of highly refractive apoptotic bodies (white arrows) in rounded cells. Raji B-cells and healthy dividing A1 cells are characterized by a “doughnut” shape in the phase contrast image, indicated by the grey arrows. Flow cytometry segregation of Raji B-cells and the bladder carcinoma transformants, as in Fig. 5, always indicated apoptosis in response to IFN- γ at the level of about 1% in the Raji B-cells, which were gated separately from the bladder carcinoma transformants based on forward and side scatter and based on eFluor labeling of the 5637 bladder carcinoma cells.