ATM & CHK-1 activity in young versus old human fibroblasts

Jessica Axelrad

Department of Environmental & Radiological Health Sciences Colorado State University

Fort Collins, Colorado 80523

ABSTRACT

ATM and CHK1 are two DNA repair proteins. As such, they are an important factor in preventing tumors caused by radiation damages and subsequent mutations in the DNA strands. As people get older their likelihood of getting cancer increases because their cells have had more opportunities to mutate. We hypothesized that it is also possible a person's chance of getting cancer increases as they get older because the DNA repair proteins, namely ATM and CHK1, become less efficient with age. Protein activity was evaluated by first damaging cellular DNA in young, middle aged, and old cells by exposing them to gamma radiation. A Western blot analysis was then performed to determine the concentration of phosphorylated (or activated) ATM and CHK1 proteins in these different aged cells. The activated ATM proteins become more concentrated the more radiation the cells are exposed to (as expected), but that, especially at the highest dose, the young and middle aged cells have a higher concentration of activated ATM than the old cells. There is a similar pattern with the CHK-1 protein blot. The activated protein concentration increases with an increase in radiation, but while the 10-G young and middle-aged cells have a significant CHK-1 concentration, the old cells do not. This decrease in the activated form may be a contributing factor to a person's increased likelihood of cancer. Not only have the old cells undergone more opportunities for a genetic mutation to occur, but the ability of the cell to repair DNA damage has decreased. To determine if this apparent decrease in activated repair proteins leads to increased DNA damage, future studies will include DNA repair assays.

INTRODUCTION

When a double stranded break (DSB) occurs in cellular DNA a pathway via many proteins, including ATM and CHK1, is initiated to repair this damage. Signalling kinases, of which ATM is one, are brought to the damage site and activated (2). These signalling kinases phosphorylate Ser-139 of histone molecules located around the DNA damage site (2). The pathway continues until transducer kinases are activated, one of which is CHK1, which then transfer the signal to p53 (2). "DNA damage responses originating from broken chromosomes or elsewhere un-repaired double-strand breaks might be part of a composite signal as cells progress towards senescence" (2). Breaking down ATM and/or CHK1 proteins in old BJ fibroblasts caused a large percentage of these cells to re-enter the S-phase of the cell cycle (2). Seluonov et al observed a 4.5 fold reduction in end joining efficiency in old versus young human fibroblasts (1). Nonhomologous end joining (NHEJ) is associated with the larger deletions and mutations seen in older cells versus the mainly point mutations seen in the younger cells (1). This phenomenon suggests that NHEJ begins to decline toward the end of presenescence and continues into when the cells stop dividing (1). This reduced efficiency may lead to persistent DSBs (1). "The role of DSB's in aging is supported by studies showing that mutations in the genes involved in DSB repair and recombination lead to accelerated aging and cellular senescence" (1).

Cellular senescence is defined by the shortening of telomere length to the point that the protein cap is no longer present and so, the uncapped telomere appears as a double stranded break. The inhibition of TERF2, a telomere bound on the loop-tail junction, may be responsible for the activation of this ATM/p53 dependent pathway because these unprotected chromosome ends appear similar to DNA double stranded breaks (DSBs) (3). Cellular senescence appears to be accompanied and actively maintained by continuous phosphorylating of DNA damage signaling kinases, such as ATM and CHK1 (2). Loss of ATM activation in human fibroblasts has been observed during the final three population doublings (2). One can conclude from this that cellular response to induced telomere dysfunction declines in fully old cells (2).

Many proteins involved in DNA repair, such as ATM, are involved in either regulation of telomeres or, in the case of ATM, signaling the cell about telomere state (3). Telomere length is related to phenotypic cellular age. After each mitotic act, the telomere length shortens (decreased number of repeats) until a critical length (5-7kb in human fibroblasts) is reached and replicative senescence begins (3). Signaling kinases, including ATM, report to the site of damage and are activated, phosphorylating histone molecules adjacent to the site of DNA damage. This also induces activation via phosphorylation of CHK1, which then transfers the signal to p53 (2). Since these uncapped telomeres present in senescent cells appear as DSB's, it can be expected that these cells would already have ATM and CHK1 activation not related to radiation damage. Thus, we used presenescent cells (cells approaching senescence but still actively dividing) for this study of DNA repair activity of radiation induced DSB's.

HYPOTHESIS

As cells age the DNA repair proteins, namely ATM and CHK-1, decrease in their ability to activate and repair DNA, thus increasing a person's risk for cancer as they age.

MATERIALS

1. 4-15% polyacrylamide gel
2. Running buffer
3. 2x loading buffer with BME
4. T-75 cell flasks
5. 37ºC CO2 incubator
6. Trypsin
7. Media (5% FBS- Fetal Bovine Serum)
8. 30 micrograms of 5c cell protein
    1. P6~ young
    2. P9~ middle aged
    3. P13~ old
9. Gamma radiation source
10. Electrophoresis power source
11. Nitrocellulose membranes (2)
12. Transfer Buffer (prepared w/ methanol & water)
4. Primary antibodies
    1. ATMpS1981 rabbit
    2. CHK-1 rabbit
5. Secondary antibodies
    1. Donkey anti-rabbit
6. 4% blotto~ 4% dry milk in 1xTBST
7. 5xTBST and 1xTBST
8. ECL Western blotting kit
9. Fluorescent scanner

METHODS

Cell growth/irradiation

5c cells were stored in liquid nitrogen until they were transferred to T75 flasks in media with 10% FBS. They were grown in a 37ºC incubator for one week, during which time their growth rates were calculated. The cells were maintained at confluence for at least four days so that they were all in the same phase of the cell cycle. They were then exposed to a specified amount of gamma radiation and harvested with trypsin after 1 hour. A lysis buffer was used to harvest the proteins from the cells and protein concentration was determined using a Lowry Protein Assay.

Western Blot Analysis

30 micrograms of each protein sample was loaded into a Western gel along with 2x loading buffer and electrophoresed for 1.5 hours at 150V. The gels were transferred overnight to nitrocellulose membranes at 40V and 0ºC. The membranes were then blocked in 4% blotto and incubated with requisite antibodies. The primary antibody incubated for 1 hour shaking at room temperature and the secondary antibody incubated for 2 hours shaking at room temperature. Wash steps in between incubations consisted of shaking membranes in 1xTBST for 10 minutes. The secondary antibody was conjugated to horseradish peroxidase. This enzyme produces luminescence when incubated with the appropriate reagents. The ECL Western blotting kit was used to develop the antibodies on the nitrocellulose membrane, and they were then taken to a fluorescent scanner (storm) to be scanned in.

Analysis

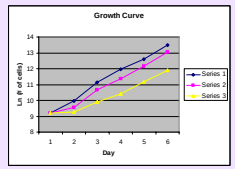
The fluorescent membranes were scanned via a storm scanner. These images were then downloaded into Image Quant Analysis where the proteins were analyzed for concentration of activated (fluorescent) protein. This volume was given a numerical value based on the pixel number and color contrast with the background, which was then graphed (shown in results).

RESULTS

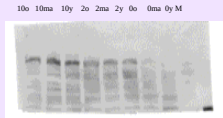
Growth Curve

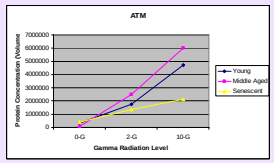
Day	Young	Series 1	Middle Aged	Series 2	old	Series 3
0	10,000	9.21034	10,000	9.21034	10,000	9.21034
1	21,160	9.959868	13,920	9.541082	10,547	9.263597
2	69,360	11.14707	43,120	10.67174	19,933	9.900132
3	155,307	11.95316	87,173	11.37565	33,267	10.41232
4	301,147	12.61535	188,160	12.14505	70,480	11.16308
5	728,227	13.49837	462,960	13.0454	147,680	11.9028

Series: ln of the number of cells. Other values are number of cells total in 5mL of media



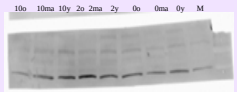
ATM

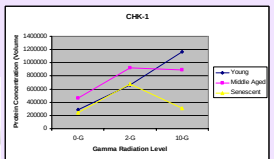




Name	Symbol	Volume	Sum	Bg. Value	Bg. Type	Percent	Average	Std. Dev.	Median
10-G old	10o	2143680	6967744	2436.396	LocalAvg	11.12	3519.063	1638.094	2827.31
10-G Middle Aged	10ma	6002899	11087108	2567.783	LocalAvg	31.13	5599.55	2737.727	5630.17
10-G Young	10y	4726087	9891751	2608.921	LocalAvg	24.51	4995.834	2736.722	4143.81
2-G old	2o	1360840	7083921	2890.445	LocalAvg	7.06	3577.738	953.411	3600.83
2-G Middle Aged	2ma	2500012	6598070	2069.726	LocalAvg	12.97	3332.358	1166.756	2969.87
2-G Young	2y	1752853	5627678	1956.982	LocalAvg	9.09	2842.261	1040.887	2569.44
0-G old	0o	407809	3320051	1470.829	LocalAvg	2.12	1676.793	204.157	1689.49
0-G Middle Aged	0ma	651979	2706222	1333.851	LocalAvg	0.34	1366.779	198.028	1344.11
0-G Young	0y	3208969	3106362	1406.814	LocalAvg	1.66	1568.87	179.353	1580.75

CHK-1





Name	Symbol	Volume	Sum	Bg. Value	Bg. Type	Percent	Average	Std. Dev.	Median
10-G old	10o	312414.5	5757762	2624.264	LocalAvg	5.55	2774.825	360.972	2689.67
10-G Middle Aged	10ma	892258.1	6990422	2938.874	LocalAvg	15.86	3368.878	362.147	3384.03
10-G Young	10y	1164066	7936683	3263.912	LocalAvg	20.69	3824.907	625.362	3718.48
2-G old	2o	679660.4	7117684	3102.662	LocalAvg	12.08	3430.209	367.492	3393.67
2-G Middle Aged	2ma	924007	7046738	2950.714	LocalAvg	16.42	3396.018	521.858	3309.03
2-G Young	2y	662190.6	6564356	2844.417	LocalAvg	11.77	3163.545	479.144	3081.4
0-G old	0o	240604.1	5812214	2685.113	LocalAvg	4.28	2801.067	280.546	2758.79
0-G Middle Aged	0ma	462261.8	5813375	2578.85	LocalAvg	8.21	2801.626	351.868	2721.96
0-G Young	0y	290090.6	5156038	2345.035	LocalAvg	5.15	2484.838	201.512	2441.04

CONCLUSIONS

We hypothesized that as cells age, the ability for the cell to repair DNA damage decreased, namely the concentration of phosphorylated (or activated) DNA repair protein, specifically ATM and CHK1, decreased. A growth curve analysis was conducted, as well as Western Blot analyses, to determine the validity of the hypothesis.

The young cells grew the fastest followed by the middle aged and then the old cells. This is relevant because, as discussed in the introduction, when DNA damage occurs, mitotic division is halted and the cells remain in the S phase of the cycle to repair the damaged DNA. The growth curve illustrates two important points. First, as the cell population ages the growth rate decreases. Second, the cells the we call "old" in this study are still growing and are therefore not senescent.

At the 0G level, the old cells actually began with a visible concentration of phosphorylated ATM. All three groups of cells increased in concentration of radiation dose, but the old cells did not increase nearly as much as the young or the middle aged. This correlates with the hypothesis that protein activity decreases with age; however, the middle aged cells showed a higher concentration of activated/ phosphorylated ATM than the young cells.

The cells investigated for CHK1 activity had a very different pattern in activity concentrations than the ATM cells. All three sets of cells began with a visible concentration, the middle aged cells being at a higher concentration than the young or old cells. The young cells were the only group that continually increased as radiation levels increased. The middle aged cells increased from 0G to 2G, but than reached a plateau from 2G to 10G. The old cells increased from 0G to 2G, but then decreased significantly (almost to their beginning level) at 10G. This data supports the hypothesis that DNA repair protein activity decreases with age.

The data supported the hypothesis. Both ATM and CHK1 proteins had a general trend of being less concentrated in older cells than in younger cells. The only discrepancy to this trend was the higher ATM concentrations in middle aged cells versus young cells. This might be due to a similar level of ATM activity in each group of cells, but more DNA damage in the middle aged cells versus the young cells. A procedure looking at damaged DNA levels would be a further step to investigate this phenomenon.

Further investigations could include conducting similar Western Blot analyses on different strains of human fibroblasts to determine if these trends are generic for human fibroblasts or specific to the 5c cells. Also, we will run gels with actin loading controls to make sure uneven gel loading is not responsible for the differences in protein concentrations. The next step would include looking at the efficiency of these repair proteins, now that we know how well or poorly they are activated in each age group.

ACKNOWLEDGEMENTS/ LITERATURE CITED

Acknowledgements to Dr. F. Andrew Ray PhD- HURS Mentor

1) Seluanov, Andrei, Mittelman, David, Pereira-Smith, Olivia M., Wilson, John H., & Gorbunova, Vera. (May 18, 2004). DNA end joining becomes less efficient and more error-prone during cellular senescence. *PNAS, 101 (20),* 7624-7629

2) von Zglinicki, T., Saretzki, G., Ladhoff, J., d'Adda di Fagagna, F., & Jackson, S. P. (2005). Human cell senescence as a DNA damage response. *Elsevier, 126,* 111-117

3) Kuimov, A. N. (2004). Polypeptide Components of Telomere Nucleoprotein Complex. *Biochemistry, 69 (2),* 117-129