Development of a Test Battery For Epigenetic Non-genotoxic Carcinogens

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Background

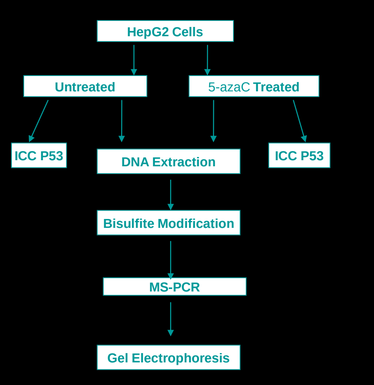
It is known that carcinogens designated on the basis of long term animal test results are extremely diverse in character, both in terms of potencies and mechanism of action, which leads to complexity in their assessment for cancer risk to humans. The classification of carcinogens into two categories, namely, Genotoxic and Non-Genotoxic varieties has been proposed to give a foundation on which cancer risk assessment can be reasonably based. The term “Genotoxic Carcinogen” indicates a chemical capable of producing cancer by directly altering the genetic material of target cells, while “Non-Genotoxic” carcinogen represents a chemical capable of producing cancer by some secondary mechanism not related to direct DNA damage (Hayashi *et al*; 1992). Epigenetic changes are thought to be associated with changes in DNA methylation and chromatin remodelling. DNA methylation seems to be the most important mechanism for “epigenetic change” at present. First, CpG methylation is inherited even after DNA replication by maintenance methylation (Araujo *et al*., 1998). Secondly CpG methylation is associated with gene silencing, and its biochemical mechanisms are being clarified rapidly (Razin *et al*., 1998). Changes in DNA methylation patterns are one of the most frequent events that occur in human tumour (Jones *et al*., 2007). In addition many non-genotoxic carcinogens are known to alter cell loss by apoptosis and many other interfere with cell to cell communication by altering the expressions of some connexins.

The main objective of this sudy is to obtain an increased insight into the mechanisms of action of epigenetic carcinogens. Although the expected number of non-genotoxic carcinogens among newly registered compounds is unknown, there is a growing concern that when numbers of 2-year cancer bioassays are significantly reduced, non-genotoxic carcinogens may go undetected. Therefore there is a need for the development of alternative methods for their detection. MS-Comet Assay seems to have a good potential to detect chemicals interfering with DNA methylation patterns, which are potential carcinogens.

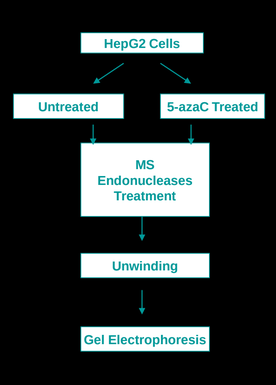
Four epigenetic carcinogens 5-azacytidine (a demethylating agent), Trichostatin A (deactylation inhibitor) Dieldrin (a tumour promoter), and Naphthalene (an anti-apoptotic agent) were selected for this study.

Methodology

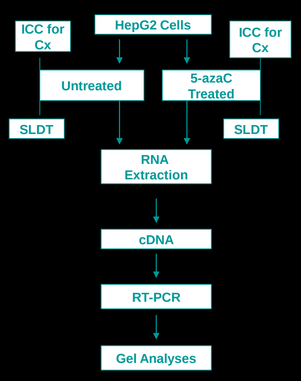
MS- PCR

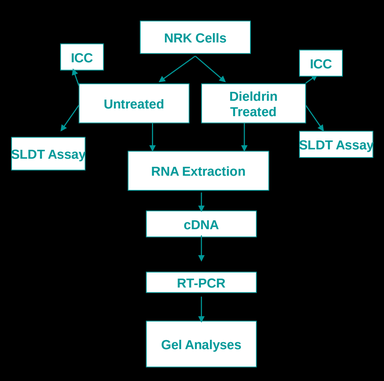


MS-Comet Assay

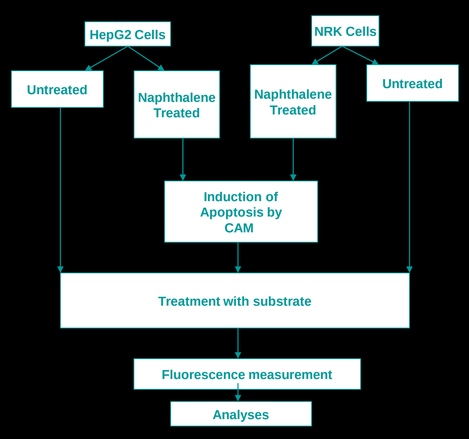


GJIC , RT-PCR, Immunocytochemistry





A study of Effects of Naphthalene on Apoptosis



Results

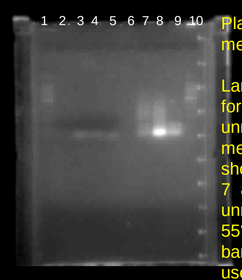


Plate 3.1. MS-PCR-based amplification of HepG2 cell p53 using methylated and unmethylated primers.

Lanes 1&10 show the 100 bp ladder. Lane 2 shows negative control for methylated primers and lane 6 shows negative control for unmethylated primers. Lane 3 & 4 show bands for p53 using methylated primers with methylated DNA (Untreated cells). Lane 5 shows a band for methylated DNA using unmethylated primers. Lane 7 & 8 show bands for unmethylated DNA (5-azaC treated) with unmethylated primers. 51°C annealing temperature for lane 7 and 55°C annealing temperature provided to lane 8. lane 9 shows a slight band for demethylated DNA as a template with methylated primers used.

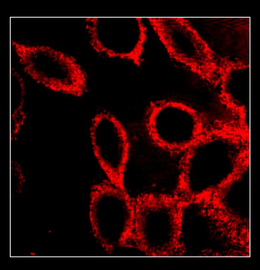


Fig 3.1 (a) Immunohistochemical detection of p53 in 5-azaC treated HepG2 cells stained with Cy5 as shown in red surrounding the nuclei stained with DAPI (not shown).

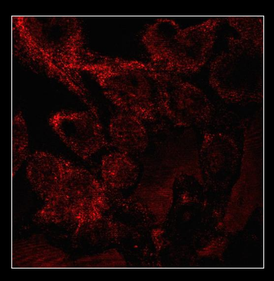


Fig 3.1 (b) Immunohistochemical detection of p53 in untreated cells stained by Cy5. Nuclei were stained with DAPI (not shown in image).

An increased expression of p53 can be seen in treated cells (Fig 3.1 a) possibly due to 5-azaC mediated demethylation of the promoter region of the p53 gene. Protein product can be seen in the cytoplasm in treated cells and in untreated it is also localized in nucleus with lower expression. One of the proteins important in the regulation of p53 is Mdm2 (murine double minute gene). Interaction of Mdm2 with p53 mediates p53 export from the nucleus to the cytoplasm. This pattern of nuclear localization of p53 has been recently attributed to the latent form of p53 in HepG2 cell lines. There might be a relationship between localization of p53 in nucleus, its migration to cytoplasm and the activation of its pathways.

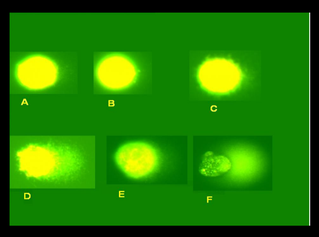


Fig 3.2(a) shows the image results for Traditional Comet Assay where (A) represents –ve control, (B) represents 5-Azacytidine treated cells, (C) represents H_2O_2 50µM (D) H_2O_2 100µM (E) 5-azac+ 50µM H_2O_2 and (F) represents 5-azac + 100 µM H_2O_2

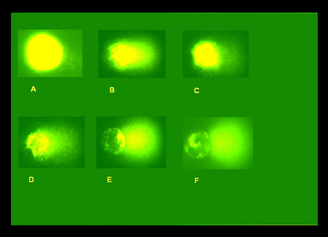


Fig 3.2(b) shows the image results for Methylation-Specific Comet Assay where (A) represents –ve control, (B) represents 5-Azacytidine treated cells, (C) represents H_2O_2 50µM (D) H_2O_2 100µM (E) 5-azac+ 50µM H_2O_2 and (F) represents 5-azac + 100 µM H_2O_2

Graph (3.1) gives a comparison of two methods which are Traditional Comet Assay and Methylation-Specific Comet Assay where Light blue columns give measurements of % tail damage for Traditional Comet Assay and dark blue columns give measurements of % tail length for Methylation Specific Comet Assay testing 5-azacytidine as a demethylating agent on HepG2 cells. it is clear from the graph that traditional Comet Assay does not report the happening of demethylation in HepG2 cells as there is no such a difference in tail length as compared to –ve control. On the other side, a clear diffrence in measurements can be obsereved between –ve control and 5-azac treated cells and more damage can be seen in this case which indicates that MS Comet Assay has the potential to report demethylating agents.

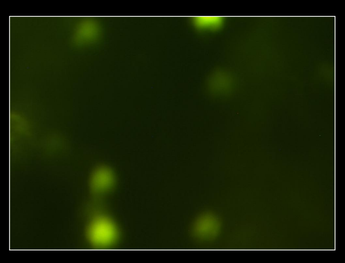


Fig 3.3(a). Dye transfer by SLDT assay in untreated HepG2 cells using Lucifer Yellow as tracer. Image was captured at 40X magnification using fluorescence microscope.

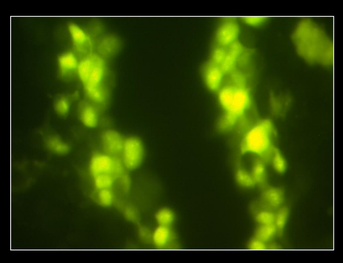


Fig 3.3(b). Dye transfer by SLDT assay in 5-azacytidine treated HepG2 cells.

Figures 3.3(a) shows that there is almost no cell to cell communication in HepG2 cells and the inhibition of the Gap Junction Communication can be clearly seen from these images as no dye transferred to the adjacent cells while on the other side in figure 3.3(b) shows a clear transfer of dye to adjacent cells. As it can be seen from the images that the distance traveled by the dye is more in figure 3.3 (b) (where cells were treated with 5-azacytidine) as compared to figure 3.3(a) where the cells were untreated. This clearly shows that 5-azacytidine has some potential to recover the communication between HepG2 cells where global hypermethylation is observed. Demethylation of some connexins and their expression has recovered the GJIC between HepG2 cells giving more like a normal phenotype as compared to untreated HepG2 cells.

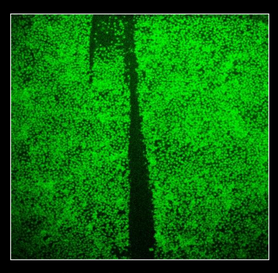


Fig3.4 (a)

NRK Untreated Cells

Fig 3.4 (b)

NRK 5 µg/ml Dieldrin treated cells

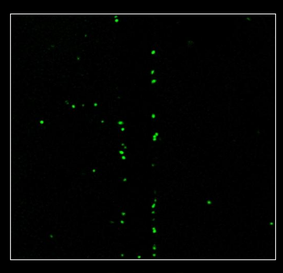


Figure 3.4 (a&b) depicts the treatment of NRK cells with the tumour promoter Dieldrin. By comparing the extent of GJIC in untreated 3.4 (a), communication proficient, NRK cells to that of NRK cells treated with Dieldrin 3.4 (b) it is clear that Dieldrin drastically down-regulates cell-cell communication between adjacent NRK cells. Indeed with Dieldrin treatment GJIC is reduced to almost non-existent levels.

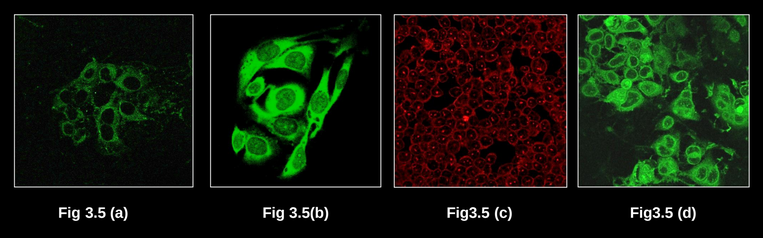


Fig 3.5 (a-d) show an immunocytochemistry analysis of connexin 26 and connexin 32 in HepG2 cells before and after treatment with 5-azaC and TSA.

Fig 3.5(a) represents localization and distribution of Cx 26 in HepG2 cells where a lower level of expression can be obsereved while after treatment with 5-azaC and TSA, an increase in the expression of Cx 26 was observed as shown in Fig 3.5 (b). Expression of Cx 32 is almost similar before and after treatment with 5-azaC and TSA. The presence and expression of Cx 32 in untreated as well treated cells was confirmed by RT-PCR as shown in Plate 3.2. The localization of Cx 32 is cell surface but the presence of Cx 26 after treatment with 5-azaC is more in cytoplasm Fig 3.5 (b) which needs to be further investigated. A different method of cell fixation for ICC for Cx 26 might need to be revived.

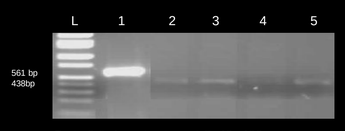


Plate 3.2. Amplification of Cx43 (438 bp) from Dieldrin treated NRK cell cDNA.

RT-PCR was also performed for Connexin 43 in untreated NRK cells as well as Dieldrin treated NRK cells. When compared to the level of expression (fluorescence intensity) of the housekeeping gene β-actin (Lanes 1) the level of expression of Connexin 43 appeared compromised in Dieldrin treated NRK cells at all concentrations tested (Lanes 3-5). However the level of expression of Connexin 43 in untreated, gap junction proficient NRK cells also appeared quite reduced (Lane 2) but a high expression was obsereved when Immunostaining was performed for untreated NRK cells

Plate 3.3. Amplification of Cx 32(361bp) from 5azaC and TSA treated HepGe2 cells cDNA

RT-PCR analysis of 5-AzaC and TSA treated HepG2 cells reveals that Cx 32 is expressed in Untreated HepG2 cells as well as in treated cells and may not have as important role in repairing GJIC as Cx 26 appears to play.

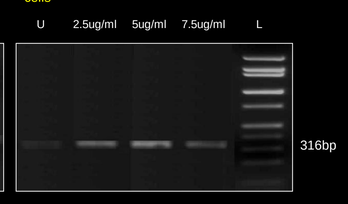
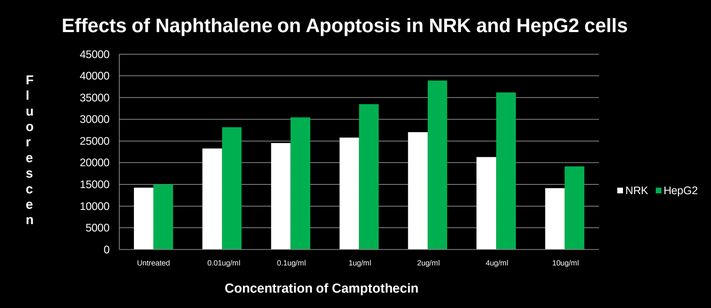


Plate 3.4. Amplification of Cx 26(316bp) from 5azaC and TSA treated HepGe2 cells cDNA

RT-PCR analysis of 5-AzaC and TSA treated HepG2 cells revealed Connexin-26 expression for all doses of 5-AzaC and TSA. More specifically at 5µg/ml 5-AzaC and 2ng/ml TSA Connexin 26 appeared to be expressed to a higher degree than at either 2.5µg/ml 5-AzaC and 2ng/ml TSA or 7.5µg/ml 5-AzaC and 2ng/ml TSA



Graph 3.2 gives a comparison of activation of caspases with the increasing concentrations of Camptothecin.
Caspase Glo 3/7 assay when performed on NRK and HepG2 cells pre-treated with Naphthalene shows that NRK cells are more resistant to CAM as compared to HepG2 cells showing a high level of Apoptosis. Naphthalene pre-treated NRK cells appear to escape apoptosis but the HepG2 do not which means Naphthalene behaves an anti-apoptotic agent for rodent cells but not for human cells.

Discussion

Different classes of non-genotoxic carcinogens behave in a variety of different ways and their mechanisms of action of these epigenetic carcinogens include tumor formation, decreased GJIC and altered expression of critical genes including cell growth, cell death and DNA repair through changes in DNA methylation pattern. DNA methylation seems to be the most important mechanism for “Epigenetic Change” at present.

Cytosine methylation has an immense importance in animal development. CpG island methylation in the promoter region of any structural gene is the known route of transcriptional silencing. Hypermethylation of cytosines in the promoter region of the p53 gene in HepG2 cells has been reported (Pogribny & James, 2002). In this study alterations in the DNA methylation status occurred in 5-azacytidine treated HepG2 cells, as detected by MS-PCR shown in plate (1) and by MS-Comet Assay as shown in Graph (1). Traditional comet assay as it is clear from the graph does not have a potential to report the demethylation as no such a difference can be seen in –ve control and 5-azacytidine treated cells. On the other side, MS-Comet Assay shows a clear difference in untreated cells and demethylated cells.

Results to date suggest that 5-azaC can upregulate the expression of p53, connexin 26 and GJIC in HepG2 cells. Conversely, Dieldrin down regulated the expression of connexin 43, and in turn GJIC, in NRK cells. Naphthalene treated NRK cells escaped apoptosis but HepG2 cells did not. This mechanism needs to be fully elucidated. Overall this study may serve as a starting point for establishing a test battery for epigenetic carcinogens.

Future Work

- To Perform the Methylation Specific Comet FISH to study the demethylation pattern in HepG2 cells after treatment with 5-azacytidine .
- To evaluate the ability of *in vitro* cell transformation assays to report non-genotoxic carcinogens

References

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