RESULTS
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Apoptosis is a phenomenon of cellular death, wherein there are specific changes in the cellular milieu, which include membrane blebbing, nuclear disintegration into apoptotic bodies and the inter-nucleosomal cleavage of DNA into nucleosomal multimers. The latter can be visualised by agarose gel electrophoresis of the DNA, as a ladder and is considered to be the hallmark of apoptosis (Kerr et al, 1972).

Glucocorticoids such as Dexamethasone, (Kerr et al, 1972), anticancer drugs or irradiation (Desozie and Sen, 1992) are some known apoptotic cell death inducers in various cellular systems. Apoptotic cell death can be induced by the interaction of a specific protein receptor on the cell and its ligand. A well-studied example is the induction of cell death by the engagement of Fas receptor with FasL, its natural ligand (Nagata et al, 1995). The Fas receptor is also capable of transmitting the death signal to the cell upon cross-linking of the Fas molecules by anti-Fas antibody, also known as anti-APO-1 (Trauth et al, 1989). Fas belongs to Tumor Necrosis Factor (TNF) superfamily and contains the death domain, which is required for the death signaling. Apart from the Fas protein, TNFR type I, as well as some of the other receptors of this family such as TNFR type II, NGFR (Nerve Growth Factor of the low affinity type) also contains the death domain and function as death signaling molecules on binding to their ligands, thereby leading to the crosslinking of the receptors.

Carcinogenesis can be envisaged as the inactivation of apoptotic pathway as well as the deregulation of cell cycle controls. Apoptosis therefore becomes an important aspect of ontogenesis of cancer as well as a target for specific anti-tumor therapy.

AK-5 tumor as the model system:

AK-5 is a rat histiocytoma with the property of spontaneous rejection and regression when injected subcutaneously in syngeneic Wistar rats (Khar et al, 1986). This property of the
tumor makes it an interesting system to study the molecular as well as immunological aspects of tumor rejection. So far, it is known that AK-5 tumor regression is a consequence of mechanisms such as ADCC (Antibody Dependent Cellular Cytotoxicity) (Khar, 1993), necrosis and NK dependent apoptosis (Kausalya et al, 1997).

The study reported here was undertaken to investigate the role of apoptosis and some of its pathways in AK-5 regression. This chapter deals with the detection of AK-5 apoptosis and purification and characterization of apoptotic factor from anti-AK-5 anti serum.

3.1.0 Purification and characterisation of apoptotic factor from anti-AK-5 antiserum

3.1.1 Induction of apoptosis in BC-8 tumor cells with anti-AK-5 anti serum:

The AK-5 cells injected subcutaneously, in syngeneic Wistar rats, grow into a solid tumor which regresses with time. The process of tumor growth and regression takes about 30 days.

The serum collected from animals which have regressed the tumor completely, is referred to as anti-AK-5 antiserum. It was first observed that BC-8 cells (a clonal population of AK-5 tumor cells adapted to grow in tissue culture conditions and have been used for all subsequent apoptosis studies reported here), when co-cultured with heat inactivated anti-AK-5 antiserum, undergo apoptotic cell death which was classified by observation of cells microscopically as well as by assaying ladder pattern of DNA due to inter-nucleosomal cleavage.

**Figure 1** shows the classical morphological features of cells undergoing apoptosis, as a phase contrast microscopy image of the apoptotic BC-8 cells on treatment with anti-AK-5 antiserum. It is observed that BC-8 cells form clumps, when treated with anti-AK-5 antiserum. There is nuclear disintegration resulting in the formation of apoptotic bodies, as observed by propidium iodide staining, shown in **Figure 2**. The DNA from apoptotic cells separates on the agarose gel as a classical ladder pattern due to inter-nucleosomal cleavage, which is considered as a hallmark of apoptosis, shown in **Figure 3**. The analysis of the
Figure 1:
Phase contrast microscope observations of:
A. Control BC-8 cells
B. BC-8 cells treated with anti AK-5 antiserum
Figure 2:

Propidium iodide staining of BC-8 cells:
A. Control BC-8 cells
B. BC-8 cells treated with anti AX-5 antiserum
Figure 3:
Agarose gel electrophoresis of DNA isolated from:
A. Control BC-8 cells
B. BC-8 cells treated with normal rat serum
C. BC-8 cells treated with anti AK-5 antiserum.
cellular DNA by FACS indicates a pre-G1 peak given by the apoptotic cells, which have less than 2N amount of DNA, as shown in Figure 4. The results discussed above clearly show that anti-AK-5 antiserum has the potential to induce apoptotic cell death in BC-8 cells, in contrast to serum isolated from normal (non-tumor injected) Wistar rats, which was used as a control.

To understand the dynamics of host-tumor interaction and apoptotic activity of anti-AK-5 antiserum, a very preliminary experiment was done where, the animals were injected subcutaneously with AK-5 tumor and serum was collected at various days post-injection/post-inoculation, and analyzed for apoptotic activity on BC-8 cells. The results are presented in Table 1 (apoptosis was scored by visual observation). These results indicate that host serum acquires the apoptosis inducing potential around day 12 post-injection of AK-5 tumor and is retained even after rejection of tumor, around day 30, strengthening the hypothesis that anti-AK-5 antiserum acquires apoptosis inducing potential due to host-tumor interaction and would contain distinct factor(s) capable of inducing apoptotic cell death in BC-8 cells.

To evaluate the specificity of apoptotic activity contained in anti-AK-5 antiserum, apoptotic assays were performed as described earlier, on various cell lines. The results are summarised qualitatively in Table 2. There was no induction of apoptosis in any of the cell lines tested, indicating that the apoptotic factor of anti-AK-5 antiserum, was specific to AK-5 tumor. These result prompted further fractionation and analysis of apoptotic activity of anti-AK-5 antiserum.

3.1.2 Strategy for purification of the apoptotic factor present in anti-AK-5 antiserum:

Anti-AK-5 antiserum was subjected to ‘heat inactivation’ to inactivate complement at 56°C for 30 min, and tested for BC-8 cell death. The cell death activity tested was therefore,
Figure 4:
FACS analysis of BC-8 cells:
A. Control BC-8 cells
B. BC-8 cells treated with anti AK-5 antiserum.
<table>
<thead>
<tr>
<th>DAY</th>
<th>APOPTOTIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>--</td>
</tr>
<tr>
<td>9</td>
<td>--</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>++</td>
</tr>
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<td>++</td>
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<td>++</td>
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<td>27</td>
<td>++</td>
</tr>
<tr>
<td>29</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 1:
Table showing apoptotic activity of serum isolated from rats on different days after the subcutaneous injection of AK-5 cells.
<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>APOPTOTIC ACTIVITY IN PHASE CONTRAST</th>
<th>APOPTOTIC ACTIVITY IN PROPIDIUM IODIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SNB19</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CAKI-1</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>RPMI</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>KM-12</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>MDAMB</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>OVICAR8</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>COLO</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>AS49</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>SW620</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>U251</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>DMB273</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>A375</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>ACHN</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>M14</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>BC-8</td>
<td>-</td>
</tr>
<tr>
<td>15 (POSITIVE CONTROL)</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2:
Table indicating the presence or absence of apoptotic cell death, in response to anti AK-5 antiserum treatment, in different cell lines.
independent of complement. The serum samples positive for apoptotic cell death were pooled and subjected to fractionation as outlined in Figure 5. The rationale followed in the purification procedure was to separate antibody and albumin fractions, which constitute the major proteins in serum, from other proteins. The fractions were tested for BC-8 cell apoptosis at each step of fractionation and the active fractions were subjected to further fractionation.

As indicated in Figure 5, the pooled serum was subjected to 45% ammonium sulphate precipitation. The supernatant was collected and the precipitate was dissolved in minimum amount of PBS. The supernatant and the precipitate were dialysed against 100 volumes of PBS for 24h after which each fraction was tested separately for apoptotic activity on BC-8 cells. The precipitate induced apoptotic cell death in BC-8 cells, while there was no cell death observed with the 45% ammonium sulphate supernatant. The precipitate was then subjected to DEAE-Sephadex ion exchange chromatography. DEAE Sephadex column was washed with 10 mM Tris-Cl pH 8.3 (as elucidated in materials and methods) and developed with a linear gradient of 0-0.5M sodium chloride. The wash and eluate fractions of DEAE-Sephadex column were collected and absorbance at 280nm (A\textsubscript{280}) was measured spectrophotometrically, which is represented as a function of fraction number in Figure 6. Subsequently, wash and eluate fractions were pooled separately, concentrated by ultrafiltration, dialysed against PBS, and tested for apoptotic activity as above. The eluate fraction was active for apoptosis, and was further separated on Protein-G affinity column.

The eluate fraction was applied on a 3ml column of Protein-G sepharose affinity matrix, which was pre-equilibrated with phosphate buffer, pH 7.0. The column was washed with phosphate buffer pH 7.0 and eluted with 100 mM glycine-HCl pH 2.7. Fractions of 1ml volume were collected and were neutralised with 50 µl of 1 M Tris-Cl, pH 9.5. Figure 7 shows absorbance at 280 nm (A\textsubscript{280}) as a function of fraction number, of the wash and eluate
STRATEGY FOR PURIFICATION OF APOPTOTIC FACTOR FROM ANTI AK-5 ANTISERUM

Collection of anti AK-5 antiserum
↓
Heat inactivation of complement at 56°C for 30 min
↓
Test for apoptotic activity and pool the active fractions
↓
45% ammonium sulphate precipitation and extensive dialysis against PBS
↓
Resolution of apoptotically active fraction on DEAE-sephadex column
↓
Dialysis and test of the wash and eluate fractions for apoptotic activity
↓
Resolution of apoptotically active fraction on a Protein G-sepharose column
↓
Dialysis and test of wash and eluate fraction for apoptotic activity

Figure 5:
Flow chart for the strategy used to isolate apoptotic factor from anti-AK-5 antiserum
Figure 6:
Protein resolution profile of 45% ammonium sulphate precipitate of anti AK-5 antiserum, resolved on DEAE-Sephadex ion exchanger, with linear NaCl gradient of 0-0.5M.
Figure 7:
Protein resolution profile of apoptotically active fraction derived after DEAE-
Sephadex ion exchange chromatography, resolved on Protein G Sepharose
affinity column.
fractions collected after Protein-G affinity column chromatography. The fractions were pooled, concentrated by ultrafiltration and dialysed against PBS. After testing for BC-8 apoptosis, it was observed that the eluate of Protein-G column was active for the induction of cell death. The activity was approximately 200 times that of the crude (data not shown). The results clearly implicate anti-AK-5 antibody fraction of anti-AK-5 antiserum as a potent apoptotic factor. The apoptotic activity profiles of fractions at each step of purification are summarized in Table 3.

To confirm the above results, the apoptotic activity was purified using various other standard protocols for purification of antibodies such as caprylic acid and sodium sulphate. The results indicate that indeed, the apoptotic factor is the antibody fraction of anti-AK-5 antiserum (data not shown).

3.1.3 Identification of the purified apoptotic factor:

The DEAE-Sephadex column eluate and the Protein-G Sephadex wash and the eluate fraction were resolved on a SDS-polyacrylamide gel using the discontinuous system as described in the materials and methods, and were detected by silver staining as shown in Figure 8. The Protein-G fraction clearly shows the 55kDa heavy chain and the 25kDa light chain bands in the presence of 2-mercaptoethanol. The 55kDa band can be seen as a doublet, probably due to differential glycosylation of the heavy chain, which is a characteristic feature of IgG-2b subtype of antibody (Harlow and Lane, 1989). In the absence of the reducing agent, 2-mercaptoethanol, the bands migrate as high molecular weight single protein indicating the presence of disulphide bonds, and in a manner characteristic of an antibody molecule as indicated in Figure 8. These results substantiate that the apoptotic factor is an antibody. Taken together, the above results clearly indicate that the autologous antibody present in anti-AK-5 antiserum, has the potential to induce apoptotic cell death in BC-8 cells.
<table>
<thead>
<tr>
<th>FRACTIONATION STAGE</th>
<th>FRACTION</th>
<th>APOPTOTIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRUDE</td>
<td>ANTI AK-5 ANTISERUM</td>
<td>++</td>
</tr>
<tr>
<td>45% AMMONIUM SULPHATE PRECIPITATION</td>
<td>SUPERNATANT PRECIPITATE</td>
<td>-- ++</td>
</tr>
<tr>
<td>DEAE-SEPHADEX ION EXCHANGE CHROMATOGRAPHY</td>
<td>WASH ELUATE</td>
<td>-- ++</td>
</tr>
<tr>
<td>PROTEIN G AFFINITY CHROMATOGRAPHY</td>
<td>WASH ELUATE</td>
<td>-- ++</td>
</tr>
</tbody>
</table>

Table 3:
Table representing the apoptotic activity of the fractions isolated after each step of fractionation of anti AK-5 antiserum
Figure 8:
SDS-PAGE and silver staining of anti AK-5 antiserum proteins at different steps of fractionation:

A. Protein G Sepharose column purified Apoptotic Factor without 2-mercaptoethanol
B. Protein G Sepharose column purified Apoptotic Factor with 2-mercaptoethanol
C. DEAE-Sephadex purified Apoptotic Factor
D. Wash fraction of Protein G Sepharose column.
E. Molecular weight markers
3.1.4 Biochemical characterization of the apoptotic factor:

The apoptotic factor was further characterized for heat stability and was found to be inactivated at around 80°C while it is stable upto 60°C (data not shown).

Protease digestion studies were done with two proteases, papain and V8. The reason for choosing papain was, to cleave the antibody at the hinge region, which would separate the Fab region from Fc, and may indicate the role of either in BC-8 cell death (BC-8 cells have FCγ receptors on the cell surface). Papain and V8 proteases were added to the purified apoptotic factor and incubated at 37°C for 2 h, and were tested for apoptotic cell death of BC-8 cells. The protease digestion, retained apoptotic activity of the factor, indicating the main role of Fab in the induction of apoptosis (data not shown).

3.1.5 Nature of interaction of purified apoptotic factor with BC-8 cells:

To characterize apoptotic factor further, experiments were done to understand the nature of its binding and interaction with BC-8 cells. Figure 9 (a, b) shows immunofluorescence of BC-8 cells fixed in 2% paraformaldehyde, where apoptotic factor is used as the first antibody, and anti-rat antibody conjugated to FITC is used as the detection system. The result indicates the binding of apoptotic factor on the surface of BC-8 cells. Figure 9b shows confocal imaging of immunofluorescent BC-8 cells.

To confirm the above results another set of experiments were done where BC-8 cells were treated with apoptotic factor for 6 h after which the cells were fixed with 2% paraformaldehyde, to crosslink the bound apoptotic factor. An anti-rat second antibody was used to detect the time dependent localization of apoptotic factor in the cells. The cells were simultaneously stained with propidium iodide to visualize nuclei. The results as shown in Figure 10 clearly indicate the presence of apoptotic factor on the surface of BC-8 cells even after 6 h and the cleavage of the nuclei into apoptotic bodies.
Figure 9a:

Immunofluorescence of BC-8 cells with Apoptotic Factor, and FITC-tagged anti rat antibody as the detection system.
Figure 9b:
Immunofluorescence of BC-8 cells with Apoptotic Factor, and FITC-tagged anti rat antibody as the detection system and with confocal imaging
Figure 10.

Double stain confocal microscopic imaging of BC-8 cells:

A. Control BC-8 cells

B. BC-8 cells treated with Apoptotic Factor for 6h; fixed and stained with FITC-tagged anti-rat antibody and propidium iodide.
3.1.6 Identification of putative BC-8 binding proteins:

To identify the cellular protein to which the apoptotic factor binds, a western analysis was done. The BC-8 cellular proteins were solubilised and resolved on a SDS-PAGE and transferred onto a nitrocellulose membrane. Apoptotic factor was used as the probe with anti-rat immunoglobulin conjugated to alkaline phosphatase as the second antibody. Figure 11 indicates that apoptotic factor binds to a protein of relative molecular weight 76kDa.

An immunoprecipitation reaction with apoptotic factor, followed by resolution of the immunoprecipitated proteins by SDS-PAGE and subsequent western of the same indicates the co-precipitation of the 76kDa protein, as indicated in Figure 12. The result indicates that the apoptotically active factor, purified from anti-AK-5 antiserum recognizes protein of relative molecular weight of 76kDa.

Taken together, the data describes a spontaneously regressing AK-5 tumor system wherein one of the mechanisms of regression is autologous antibody induced apoptosis of the tumor cells. The antibody binds to the tumor cell surface and recognizes a tumor cellular protein of 76kDa relative molecular weight.

The antibody induced apoptotic cell death is a well known phenomenon for the cell surface receptors of the TNFR superfamily such as TNFR type I and type II, NGFR (low affinity receptor) and Fas receptor or anti-IgM induced cell death of the B-cells through BCR (B-cell receptor). All these receptors require their crosslinking through either the physiological ligand or by an anti-receptor antibody. Particularly, the transmission of death signal by FAS upon crosslinking with anti-APO-1 monoclonal antibody (Trauth et al, 1989) is a well-studied system.

It has been observed that FasL the physiological ligand for Fas is present on AK-5 tumor cells (Khar et al, 1999) but neither Fas protein nor Fas message was detected in western or in northern analysis, which indicate the absence of Fas induced cell death in AK-5 system. Also,
Figure 1b:

Western analysis of BC-8 cellular proteins with Apoptotic Factor and alkaline phosphatase conjugated anti-rat antibody:

A. Treated with Apoptotic Factor and anti-rat antibody

B. Control (treated with anti-rat antibody)
Figure 12:

Immunoprecipitation of BC-8 cellular proteins with:

A. Protein G beads (pre-cleared)
B. Normal rat antibody
C. Apoptotic Factor (anti AK-5 antibody); Western analysis of the immunoprecipitate proteins with Apoptotic Factor (anti AK-5 antibody) as the probe.
there does not seem to be any role of the TNFα in AK-5 cell death, as the serum factor active for apoptosis is devoid of TNFα, which was measured by L929 cell line sensitivity assay (data not shown). Since the antibody is autologous (from syngeneic animals) and did not induce any apoptosis in the battery of cell lines tested, the antibody would be against a protein specific to AK-5 tumor, wherein lies the novelty of this system. The putative apoptotic factor binding protein was identified from Western analysis as well as from immunoprecipitation experiments to be of 76kDa relative molecular weight. The two other protein bands in the immunoprecipitation could be due to co-immunoprecipitation of other proteins along with the 76kDa protein. The protein is as yet unidentified and N-terminal sequencing data may give more insights into the apoptotic mechanism.
3.2.0 Signal transduction mechanisms utilised by apoptotic factor to induce apoptotic cell death:

3.2.1 Phosphorylation:

Phosphorylation of proteins at the amino acids tyrosine, serine or threonine is known to be one of the most important signaling mechanisms in the eukaryotic cellular systems. It is known that phosphorylation plays an important role in the apoptotic mechanisms as well. Kinases which are of ser/thr class as well as that of tyr kinases play an important role in the cell survival and cell death mechanisms.

3.2.2 Phosphorylation of BC-8 cell proteins after the interaction of apoptotic factor:

The purpose of conducting these experiments was to understand the downstream pathways by which apoptotic factor mediates apoptosis in BC-8 cells. It is known that the apoptotic death machinery and its mechanisms are conserved from C.elegans to humans, though the initial signals could be different. Here an attempt was made to identify the initial signaling pathways, which would provide an insight into the nature of the 'receptor' protein to which apoptotic factor interacts with.

The phosphorylation experiments were done in vitro, with γP^{32}ATP, after the cells were incubated with apoptotic factor, to trigger the activation of kinase(s), which could utilize γP^{32}ATP to phosphorylate substrate proteins. The proteins were resolved on SDS-PAGE and analysed by autoradiography.

As shown in Figure 13, there a difference in the phosphorylation pattern of AK-5 cells treated with the apoptotic factor as against the normal rat serum antibody control as well as that of Jurkat T cell line control. It was observed that there is no induction of apoptosis by apoptotic factor in the Jurkat T cells when tested under conditions similar to that of BC-8 cells, which were, therefore, used in control studies. Figure 13 indicates the phosphorylation
Figure 13.
Protein phosphorylation with $\gamma^32$ ATP:
A. Untreated BC-8 cells
B. BC-8 cells treated with normal rat antibody
C. BC-8 cells treated with Apoptotic Factor (anti AK-5 antibody)
D. Untreated Jurkat cells
E. Jurkat cells treated with normal rat antibody
F. Jurkat cells treated with Apoptotic Factor (anti AK-5 antibody)
G. Jurkat cells treated with anti-Fas antibody.
of three proteins of relative molecular sizes 94kDa, 78kDa and 73kDa. These results also indicates that the pattern of phosphorylation is specific to the interaction between apoptotic factor and BC-8 cells, as compared to Jurkat T cells, which have Fas receptor and do not show the same pattern. This indicates that apoptotic factor mediated cell death does not engage the Fas protein as the primary death inducing receptor. This data is corroborated elsewhere, where it is shown that Fas message is undetectable in BC-8 cells.

3.2.3 Time dependent phosphorylation of BC-8 cell proteins after interaction with apoptotic factor:

Figure 14 shows the increase in the phosphorylation of these proteins with the increase in the time of incubation of the apoptotic factor with AK-5 cells. The time of incubation were 0min (instantaneous), 10, 20, 30 min. Figure 15 shows the densitometric profile of the three proteins, which indicates a graded increase in the phosphorylation of these proteins. These results indicate that there is an immediate and sustained occurrence of phosphorylation events, due to the interaction of apoptotic factor with BC-8 cell surface.

3.2.4 Determination of the nature of the phosphorylated amino acid:

To study whether the phosphorylation of the proteins was on tyrosine or on serine or threonine of these proteins, the resistance to alkali hydrolysis of the proteins was determined. Treatment with 1 N NaOH, at 65°C for 1h, and subsequent autoradiography, indicated the disappearance of 78kDa and 73kDa protein bands, as shown in Figure 16. This indicates the specific phosphorylation of serine or threonine amino acids of these proteins and the possible activation of a serine/threonine kinase.
Figure 14:
Time dependent phosphorylation pattern of proteins after treatment with Apoptotic Factor for different times: U (untreated), 0, 10, 20 and 30 min.
Figure 16:
Alkali hydrolysis profile of the phosphorylated proteins at various time points: U (untreated, control) 0, 10, 20 and 30 min.
3.2.5 Study of phosphorylation profiles with specific kinase inhibitors:

To elucidate further the role of kinases in apoptotic factor induced BC-8 phosphorylation and apoptosis, kinase inhibitors such as Herbimycin A, Genestein and Staurosporine were used. Herbimycin A did not inhibit phosphorylation at various concentrations; (the result could mean that tyrosine phosphorylation was not important for apoptosis; but such a result could also indicate a 'false negative' if Herbimycin A had lost its tyrosine kinase inhibitory activity, and therefore needs to be interpreted with caution). On the other hand, Staurosporine inhibited the phosphorylation of the 78 and 73kDa bands at a concentration of 5nM. Even though, staurosporine is a wide range kinase inhibitor, it is specific for serine/threonine kinases (like protein kinase C) at the concentrations of 3-6 nM (Tamaoki, 1991). The result, as shown in Figure 17, therefore implicates the activation of serine/threonine kinase(s) in response to apoptotic factor and BC-8 cell surface interaction.

3.2.6 Correlation of phosphorylation with apoptotic factor induced BC-8 apoptosis:

To understand the relevance of serine/threonine phosphorylation and its role in BC-8 apoptosis, the induction of apoptosis in BC-8 cells by apoptotic factor, in the presence of 5 nM staurosporine, was studied. Table 4 shows the percentages of BC-8 cells undergoing apoptosis with and without staurosporine, as quantitated by FACS analysis. The results in Table 4 clearly indicate the inhibition of apoptosis in the presence of 5nM staurosporine. (Higher concentrations were toxic to the cells—data not shown)

These results indicate a unique phosphorylation pattern in BC-8 cells in the presence of apoptotic factor. The phosphorylation occurs within the first few minutes, and is sustained. Further, this phosphorylation signal is required for BC-8 cell death as indicated by the kinase inhibitor, staurosporine data. The nature of the proteins phosphorylated is not known, but cannot be MAP kinases, as indicated by their size. A conjecture could be made that these
Figure 17:
Effect of protein kinase inhibitors, herbimycin A, and staurosporine on protein phosphorylation induced by ApoptoticFactor:
A. Untreated, control BC-8 cells
B. BC-8 cells treated with apoptotic factor
C., D., E. BC-8 cells treated with herbimycin A 100 μg 150 μg, 250 μg
F. BC-8 cells treated with staurosporine 5nM
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PERCENTAGE OF PRE-G0/1 CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNTREATED BC-8 CELLS</td>
<td>1.15</td>
</tr>
<tr>
<td>CELLS TREATED WITH ANTI AK-5 ANTISERUM</td>
<td>22</td>
</tr>
<tr>
<td>CELLS TREATED WITH 5nM STAUCROSPORINE AND ANTI AK-5 ANTISERUM</td>
<td>6.58</td>
</tr>
</tbody>
</table>

Table 4: Percentage inhibition of apoptotic cell death of BC-8 cells treated with Apoptotic Factor in the presence of 5nM staurosporine
proteins are the initial signaling molecules, transducing the death signal initiated by the binding of apoptotic factor to the surface protein.

3.3.0 Role of calcium as one of the signal transducers:

It is a well-known that calcium acts as an important second messenger in signal transduction pathways. It is also known that in apoptotic cell death pathways, calcium activates a calcium dependant endonuclease (Cohen et al, 1984) which cleaves the DNA into nucleosomal multimers. Some of the apoptotic pathways utilize calcium while others are independent. It was, therefore, interesting to know whether apoptotic factor mediated BC-8 cell death pathway could trigger calcium flux in these cells, and the nature of such a change.

The study was performed using INDO-1-AM, a calcium binding fluorescent dye, the emission of which can be monitored at two wavelengths, 485nm for the unbound dye and at 405nm for dye saturated with calcium. The excitation is at 351-356 nm.

BC-8 cells were loaded with INDO-1-AM dye at a concentration of 10μM/ml. The cells were washed, suspended in plain medium and cells were monitored individually for differences in the calcium bound dye intensity to calcium free dye intensities (Intensity at 485nm : Intensity at 405nm). The ratio is plotted as a function of time. The experiment was done after the cells were treated with apoptotic factor and with dexamathasone. Dexamethasone was used as a comparative control to detect a difference in the kinetics of the flux. Figure 18 indicates the change in ratio of intensities of calcium bound INDO-1 to calcium free INDO-1, as a function of time, after treatment with dexamethasone (0.2mg/ml), as well as with pretreatment of the cells with EDTA. Figure 18 shows the increase in calcium flux to a peak level, after treatment with dexamethasone in the first 1 min, which can be inhibited by EDTA. The results indicate that calcium flux triggered with dexamethasone occurs within the first one minute, indicating that the kinetics are relatively fast and it is the external calcium which enters the cell, as the
Calcium flux in BC-8 cells in response to dexamethasone

Figure 18:
Calcium flux as monitored by INDO-1-AM fluorescence and confocal microscopy, in response to dexamethasone treated and untreated with EDTA.
flux is inhibited by the addition of EDTA. The confocal microscopic image of cells undergoing calcium flux can be seen in Figure 19.

Figure 20 indicates, the ratio of calcium bound dye to dye free of calcium as a function of time after treatment with apoptotic factor. The kinetics of calcium flux are slow, and begin to peak after 10 minutes. The calcium flux is, as indicated by the inhibition with EDTA, through the external medium. Figure 21 indicates the confocal image of the cells undergoing calcium flux. Calcium flux when monitored in calcium free PBS (data not shown), does not show any change corroborating the evidence that external calcium enters the cell in response to both dexamethazone and apoptotic factor, although the kinetics of both these fluxes are different. Further, in case of apoptotic factor the phosphorylation events precede calcium flux since the former events are instantaneous while the latter occur only after the first 10 minutes. The observation can be explained with a conjecture that apoptotic factor requires its binding to a putative 'receptor' which then transduces the signal.
Figure 19:
Calcium flux detection by confocal microscopy of BC-8 cells with INDO-1-AM:
A. Control BC-8 cells
B. BC-8 cells treated with dexamethasone (0.2mg/ml) for 1 min
Calcium flux in BC-8 cells in response to Apoptotic Factor

![Graph showing calcium flux in BC-8 cells](image)

- Treated with Apoptotic Factor (Anti AK-5 antibody)
- Treated with EDTA and Apoptotic Factor

**Figure 20:** Calcium flux in BC-8 cells, in response to Apoptotic Factor in presence and absence of EDTA as monitored by INDO-1-AM fluorescence and confocal microscopy.
**Figure 21:**

Calcium flux detection by confocal microscopy of BC-8 cells with INDO-1-AM:
A. Control BC-8 cells
B. EC-8 cells treated with Apoptotic Factor (anti AK-5 antibody) for 12 min.
3.4.0 Role of proto-oncogenes c-myc and c-jun in apoptotic factor induced apoptosis:

Proto-oncogenes such as c-myc, c-jun, etc., are deregulated as a result of the activation of apoptotic pathway. It is also known that overexpression of c-myc leads to apoptotic cell death. The present study, therefore, was conducted to understand the role of c-myc proto-oncogene in the apoptotic factor from the serum induced apoptotic cell death in BC-8 cells.

RNA isolated from the control BC-8 cells and those treated with either anti AK-5 antiserum or apoptotic factor, were resolved on a 1% agarose gel and transferred on to a nylon membrane. The membrane was blocked and treated with the c-myc probe labeled with $\alpha$P$^{32}$ATP by either a random priming or nick-translation reaction.

Figure 22(A) indicates decrease in mRNA levels of c-myc in the cells treated with either apoptotic factor or anti AK-5 antiserum for 3 h. Figure 22(B) indicates the GAPDH levels to show equal loading of RNA samples. Further, northern hybridizations done with RNA isolated from BC-8 cells treated with apoptotic factor from the serum for various time intervals indicate that this decrease in c-myc mRNA levels can be detected by 1h of treatment of apoptotic factor from the serum as shown in Figure 23. The corresponding GAPDH levels are shown in Figure 23. These results indicate that apoptotic factor from the serum induces deregulation of proto-oncogene c-myc message in BC-8 cells and could be one of the events leading to apoptotic cell death. This event could be placed downstream of other events such as phosphorylation and calcium flux as indicated by their time intervals (Figure 23). To further ascertain if c-myc deregulation was directly in the apoptotic pathway, experiments were done to block apoptotic cell death by various methods and study the changes in c-myc mRNA levels. The results of such experiments are discussed below.
Figure 22:
Northern analysis of c-myc proto-oncogene:
A. Northern hybridization of c-myc with RNA isolated from BC-8 cells
B. Same blot hybridized with GAPDH for the normalization of RNA levels
a. control BC-8 cells
b&c. BC-8 cells treated with anti AK-5 antiserum for 3h
Figure 2

Time dependent analysis of c-myc RNA levels:

RNA isolated from BC-8 cells treated with anti Ak-5 antiserum for 0h, 30 min, 1h, 2h, and 3h; the same blot was hybridized with GAPDH; 2kb band indicates c-myc and 1.6kb band indicates GAPDH
3.4.1 Role of caspases in anti AK-5 anti serum induced BC-8 apoptosis and the
deregulation of c-myc mRNA:

Northern hybridisations for c-myc were done as above, with a clonal population of BC-8
cells transfected with caspase-2 antisense. These cells are resistant to apoptotic factor from
the serum induced apoptosis. The northern analysis indicates no change in c-myc mRNA
levels and is shown in Figure 24. These results indicate a role of caspases in the deregulation
of c-myc proto-oncogene during apoptosis. To test this hypothesis further, BC-8 cells were
pre-treated with specific caspase inhibitors such as DEVD-CHO and YVAD-CHO for 30
min, and then treated with apoptotic factor from the serum, for 3 h. The mRNA was isolated
and northern analysis done. The results are shown in Figure 24. Here too, there is no change
in the levels of mRNA, which were pre-treated with the peptide caspase inhibitors DEVD-
CHO and YVAD-CHO. These results indicate the role of caspases: CASPASE-2, CASPASE-
1 and CASPASE-3 in the apoptotic programme activated by apoptotic factor from the serum,
upstream to that of c-myc regulation. This is an interesting finding and indicates that there
could be a protein(s) involved in transcription of c-myc mRNA, which is the target of
caspases. Alternatively, the caspases could be acting on protein(s) involved in the stability of
c-myc mRNA, since it is known that c-myc regulation occurs mainly at post-transcriptional
level and mRNA stability is an important factor.

3.4.2 Role of bcl-2 in the deregulation of c-myc expression:

Northern analysis conducted on the RNAs isolated from BC-8 cells transfected with bcl-2
gene, and resistant to apoptotic factor from the serum induced apoptosis, indicate no decrease
in the c-myc mRNA levels between those treated and untreated with anti AK-5 anti serum as
shown in Figure 25. These results are in concordance with the results known from other
studies, which show that caspases act downstream to the BCL-2 protein and are activated by
Figure 24:

Northern analysis of c-myc on:

a. control RNA from BC-8 cells transfected with caspase-2 antisense
b. Caspase-2 transfected BC-8 cells and treated with anti AK-5 antiserum for 3h
c. BC-8 cells pre-treated with DEVD-CHO and treated with anti AK-5 antiserum for 3h

BC-8 cells pre-treated with YVAD-CHO and anti AK-5 antiserum for 3h; 2kb indicate c-myc hybridisation, and 1.6kb indicates GAPDH hybridisation.
Figure 25:
Northern analysis of c-myc on bel-2 transfected BC-8 clones:
A. Hybridization with c-myc
B. Hybridization with GAPDH

a. control BC-8 cells transfected with bel-2
b. BC-8 cells transfected with bel-2 treated with anti AK-5 antiserum for 3h.
the release of cytochrome C due to the increase in mitochondrial potential, where BCL-2 activity is supposed. Our data, therefore, indicates an upstream action of BCL-2 and caspases to the deregulation of c-myc.

Similar experiments indicate the decrease in c-jun mRNA levels, after 3 h of treatment with apoptotic factor from the serum. These results as shown in Figure 26(A) and Figure 26(B) indicate the GAPDH mRNA levels for normalization. This deregulation can be reversed in the presence of DEVD-CHO and YVAD-CHO, specific peptide caspase inhibitors as shown in Figure 27. These results also point to a generalized regulation of mRNAs such as c-myc and c-jun through caspases. It is known that caspases activate certain nucleases and a conjecture could be made that these nucleases could play an important role in regulation of these genes.

3.4.3 Relation of calcium flux to c-myc and c-jun deregulation:
BC-8 cells are treated with apoptotic factor from the serum in calcium free medium for 3 h and RNA is isolated as above. Northern hybridisations are done with c-myc and c-jun probes. Figure 28 indicates that there is no change in the c-myc mRNA levels. The result indicates the importance of calcium in the c-myc deregulation. Further, Figure 29 indicates that c-jun mRNA levels are unaffected as well when BC-8 cells are treated with apoptotic factor from the serum in the absence of calcium. The results indicate the upstream action of calcium, which would be expected if it were acting as a second messenger.

3.4.4 Role of other genes:
The levels of other mRNA such as waf-1, FCRγ (CD25), p53 do not indicate any difference in levels from treated and control (untreated) samples (data not shown). It is known that there
Figure 26.
Northern analysis of c-jun proto-oncogene.
A. RNA blot hybridised with c-jun probe
B. same blot hybridised with GAPDH for normalisation
a. BC-8 control total RNA
b. BC-8 control mRNA
c. BC-8 cells treated with anti AK-5 antiserum mRNA
d. BC-8 cells treated Apoptotic Factor mRNA.
Figure 27:

Northern analysis of *c-jun* on RNA isolated from BC-8 cells treated with caspase inhibitors DEVD-CHO and YVAD-CHO and anti AK-5 antiserum:

A. Blot hybridised with *c-jun*

B. Same blot hybridised with GAPDH

a. RNA from BC-8 cells treated with DEVD-CHO and anti AK-5 antiserum

b. RNA from BC-8 cells treated with YVAD-CHO and anti AK-5 antiserum.
Figure 28:
Northern hybridisation with c-myc in the absence of calcium:

A. Hybridisation with c-myc
B. Hybridisation with GAPDH

a. control BC-8 cells in calcium free medium
b. BC-8 cells treated with anti AK 5 antiserum in calcium free medium
Figure 29:
Northern hybridisation with c-jun in the absence of calcium:
A. Hybridisation with c-jun
B. Hybridisation with GAPDH
a. control BC-8 cells in calcium free medium
b. BC-8 cells treated with anti-AK-5 antiserum in calcium free medium.
is an increase in p53 levels, in apoptosis induced by radiation. But in this case, the apoptosis
does not seem to be induced by p53.

Known factors such as Fas, do not play a role in anti AK-5 antibody induced apoptosis of
BC-8 cells. As shown in Figure 30(A), Fas message is undetectable as compared to its level
in thymocyte RNA. Figure 30(B) shows the GAPDH levels, which indicate an excess of
mRNA loaded in BC-8 lane.
Figure 30:

Northern hybridisation with *fas*:
A. Hybridisation with *fas*
B. Hybridisations with GAPDH
a. mRNA isolated from BC-8 cells
b. mRNA isolated from thymocytes (positive control)