Assessment of anti-neoplastic activity in cancerous and non-cancerous cells using nuclear growth indicators

James Michael Williams
ASSESSMENT OF ANTI-NEOPLASTIC ACTIVITY
IN CANCEROUS AND NON-CANCEROUS CELLS
USING NUCLEAR GROWTH INDICATORS

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
James Michael Williams
December 1997
ASSESSMENT OF ANTI-NEOPLASTIC ACTIVITY
IN CANCEROUS AND NON-CANCEROUS CELLS
USING NUCLEAR GROWTH INDICATORS

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

by
James Michael Williams
December 1997

Approved by:
Klaus Brasch, Chair, Biology
Richard Fein
Jeffrey Thompson
James Ferrari

Dec. 8, 1997
ABSTRACT

It is well known that many plants, algae, and microorganisms contain or secrete agents which may exhibit anti-cancer or cell growth modulating properties. Of these, extracts from Chara globularis Thuill, a fresh water alga, may contain an agent(s) which exhibits such properties. Experiments were designed to address the question, does the potential growth inhibitory properties of Chara, alter specific nuclear growth indicators in cancerous and non-cancerous cells, and if so what might this indicate about the potential mechanism of Chara extract? From this question, the hypothesis that Chara modulates the appearance of nuclear growth indicators related to transcription and cell-cycle in Jensen sarcoma but not in non-cancerous cells was derived. In this study, the effects of Chara extracts were compared to a well characterized anti-neoplastic agent, doxorubicin.

The cancerous cell line, Jensen sarcoma, which has been shown sensitive to treatment with extract from Chara both in vivo and in vitro, was compared to the non-cancerous cell lines, Chang liver and (embryonic) Intestine 407 cells. The effects of Chara extract and doxorubicin on cells were assessed by immunofluorescence using antibodies that recognize the following nuclear proteins: fibrillarin, p-80 coilin, and proliferation cell nuclear antigen (PCNA). Fibrillarin, a nucleolar protein associated with U3 small nuclear ribonucleoprotein (snRNP) and involved in rRNA processing, may provide information on the integrity of nucleolar processes. P80-coilin is a unique protein that is associated with nuclear structures referred to as coiled bodies. Coiled bodies (CBs) are most prominent in hormonally stimulated tissues and cancer cells. Their frequency has been correlated with high
receptor levels and enhanced transcription in several steroid-hormone target tissues. PCNA is a cell-cycle dependent protein, associated with DNA polymerase delta. During DNA replication, PCNA becomes associated with replication sites and exhibits a granular labeling pattern. Using these antibodies, information about the action of the anti-neoplastic agents on transcriptional and cell-cycle processes were monitored. Treatment of Jensen sarcoma with 5% v/v Chara extract or 5nM doxorubicin for 6 days was sufficient to alter the rate of growth and reduce cell density by 50%. Similarly, the rate of growth and cellular density of Intestine 407 cells appeared to lowered by treatment with doxorubicin. In contrast, Chara extract did not alter the rate of growth of either Chang liver or Intestine 407, but did depress cell density near the plateau phase.

Although neither Chara extract or doxorubicin seemed to greatly affect nuclear structures containing fibrillarin, major differences were apparent in CB frequency with respect to cell type, time, and treatment. In Jensen sarcoma, coiled body frequency was sharply decreased after treatment with either agent, relative to control cells. Chara extract did depress the CB frequency in the non-cancerous cells as well, but this effect was less dramatic than observed in Jensen sarcoma. Additionally, the effect Chara extract on CB frequency was less, compared to doxorubicin treated intestine 407 cells. Assessment of cell-cycle parameters using antibodies against PCNA showed that Jensen sarcoma treated with either Chara extract or doxorubicin exhibited significant changes in the PCNA labeling distribution compared to controls. These changes were more dramatic in Chara extract treated Jensen sarcoma compared to doxorubicin. Although, non-cancerous cells treated with either agent
did exhibit some changes, these effects were not as
dramatic as seen in Jensen sarcoma. Collectively, these
observations revealed a complex pattern of change in
nuclear transcriptional and cell-cycle activity with
respect to time, cell type and treatment. Additionally,
these observations suggests that Chara extract does appear
to contain agent(s) which exhibit a selective cytostatic
activity against Jensen sarcoma compared to the non-
cancerous cells.
First off I would like to thank my committee members, Dr. Richard Fehn, Dr. Jeffrey Thompson and Dr. James Ferrari for their patience during the production of this manuscript along with their rapid review. I would also like to thank my advisor Dr. Klaus Brasch for taking me into his lab and allowing me the opportunity to carry out this project. His guidance, support and patience during this project and preparation of this manuscript was invaluable and can not be expressed enough.

In addition I would like to thank Dr. Dalton Harrington for freely providing his insight about Chara globularis. Similarly, I would like to thank Dwight Gallo for teaching me various cell culture techniques along with helping in the preparation of the Chara extract. Without his assistance this project may never have begun in the first place.

Also I would like to thank my wife Rhonda Williams and my daughter Brittny Williams for their support and understanding during this project along with the completion of this manuscript. This work was supported by NIH grant 1R15AI32691.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Biological Properties of <strong>Chara</strong></td>
<td>1</td>
</tr>
<tr>
<td>Functional Organization of the Cell Nucleus.</td>
<td>3</td>
</tr>
<tr>
<td>A. Overview of Nuclear Organization</td>
<td>3</td>
</tr>
<tr>
<td>B. Nuclear Bodies and Coiled Bodies</td>
<td>7</td>
</tr>
<tr>
<td>C. Proliferating Cell Nuclear Antigen (PCNA)</td>
<td>10</td>
</tr>
<tr>
<td>METHODS</td>
<td>11</td>
</tr>
<tr>
<td>Project Overview</td>
<td>11</td>
</tr>
<tr>
<td>A. Cell Lines Characterization</td>
<td>11</td>
</tr>
<tr>
<td>B. Doxorubicin</td>
<td>11</td>
</tr>
<tr>
<td>C. Experimental Design and Goals</td>
<td>12</td>
</tr>
<tr>
<td>D. Experimental Analysis</td>
<td>14</td>
</tr>
</tbody>
</table>

vii
Procedures .................................................. 15

A. Cell Cultures .......................................... 15

B. Preparation of Anti-neoplastic Agents ........ 16

C. Preparation of Culture Media .................... 17

D. Cellular Proliferation ............................... 17

E. Immunological Techniques .......................... 17

F. Western Blotting ....................................... 19

RESULTS ....................................................... 21

Preparation of Chara Extract .......................... 21

Effects of Agents on Cell Proliferation .......... 21

Morphological Changes ................................. 22

Effects of Agents on Nuclear Indicators .......... 29

A. Fibrillarin ............................................. 29

B. Coiled Bodies ......................................... 35

C. Coiled Body Subpopulation ....................... 41

D. Western Blot Analysis ............................... 52

E. PCNA Quantification .................................. 52
<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th>69</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Proliferation and Morphology</td>
<td>69</td>
</tr>
<tr>
<td>Fibrillarin Containing Structures</td>
<td>71</td>
</tr>
<tr>
<td>Coiled Bodies and Cell-Cycle</td>
<td>72</td>
</tr>
<tr>
<td>Additional Modes of Action for Chara extract Components</td>
<td>77</td>
</tr>
<tr>
<td>Conclusion</td>
<td>78</td>
</tr>
</tbody>
</table>

| REFERENCES | 80 |
LIST OF TABLES

Table 1. Pairwise comparison of the influence of time on the average number of fibrillarin labeled structures for each cell type within control, Chara and doxorubicin ........... 34

Table 2. Pairwise comparison of the influence of time on the average number of p80-coilin containing structures for each cell type within control, Chara and doxorubicin ........... 40

Table 3. Pairwise comparison of the influence of time in Jensen sarcoma within control, Chara extract and doxorubicin treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs . . 44

Table 4. Pairwise comparison of the influence of time in Intestine 407 within control, Chara extract and doxorubicin treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs . . . . 47

Table 5. Pairwise comparison of the influence of time in Chang liver within control and Chara extract treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs . . . . . . 50

Table 6. Relative optical densities in western blot of p80-coilin using the human antibody Shiptak . . 55

Table 7. Pairwise comparison of the influence of time in Jensen sarcoma within control, Chara extract and doxorubicin treated cells for number of nucleolus-negative, granular and homogenous labeled cells . . . . . . . . . . . . 61

Table 8. Pairwise comparison of the influence of time in Intestine 407 within control, Chara extract and doxorubicin treated cells for number of nucleolus-negative, granular and homogenous labeled cells . . . . . . . . . . . . 64
Table 9. Pairwise comparison of the influence of time in Chang liver within control and Chara extract treated cells for number of nucleolus-negative, granular and homogenous labeled cells ................. 67
LIST OF FIGURES

Figure 1. Diagram illustrating the major components of a mammalian interphase nucleus .......................... 4

Figure 2. Logarithmic growth curves of Jensen sarcoma, Intestine 407 and Chang liver cells as assessed by trypan blue exclusion following dissociation with trypsin and EDTA ........ 23

Figure 3. Logarithmic growth curves of Jensen sarcoma, Intestine 407 and Chang liver cells as assessed by trypan blue exclusion ........ 25

Figure 4. Phase contrast photomicrographs of control, Chara extract and doxorubicin treated cells from Jensen sarcoma, Intestine 407 and Chang liver cells .......... 27

Figure 5. Photomicrographs of control, Chara extract and doxorubicin treated cells from Jensen sarcoma, Intestine 407 and Chang liver cells labeled with mouse monoclonal antibody 72B9 against the nuclear protein fibrillarin ........ 30

Figure 6. Quantitation of Fibrillarin containing nuclear structures in Jensen sarcoma, Intestine 407 and Chang liver .......... 32

Figure 7. Photomicrographs of control, Chara extract and doxorubicin treated cells from Jensen sarcoma, Intestine 407 and Chang liver cells labeled with rabbit antibody R288 against the protein p80-coilin ........ 36

Figure 8. Quantitation of p80-coilin containing coiled bodies in Jensen sarcoma, Intestine 407 and Chang liver ........ 38

Figure 9. Quantitation of coiled body subpopulation in Jensen sarcoma .......... 42

Figure 10. Quantitation of coiled body subpopulation in Intestine 407 .......... 45
Figure 11. Quantitation of coiled body subpopulation in Chang liver ............... 48

Figure 12. Western blot using the human antibody Shiptak against p80-coilin in Jensen sarcoma, Intestine 407 and Chang liver .......... 53

Figure 13. Photomicrographs of human antibody AK against PCNA in untreated and treated Jensen sarcoma, Intestine 407 and Chang liver ...................... 56

Figure 14. Quantitation of PCNA labeling in Jensen sarcoma ................. 59

Figure 15. Quantitation of PCNA labeling in Intestine 407 ................. 62

Figure 16. Quantitation of PCNA labeling in Chang liver ................. 65

xiii
INTRODUCTION

In the continuing search for new drugs which exhibit anti-cancer and cell growth inhibitory properties, interest has increasingly focused on natural products. Extensive studies along these lines have resulted in the discovery of several natural compounds with promising potential. (For Review: Slichenmyer and Von Hoff, 1990). Among these, Taxol, isolated from Western yew bark, has received much attention recently and is undergoing extensive clinical testing. Fresh water algae from the genus Chara may also contain agents with cancer growth inhibitory properties. What follows is a review of Chara's biological properties in terms of cell growth inhibition, as well as a review of literature related to this project as a whole.

Biological Properties of Chara

Caballero (1919) first reported on potential mosquitocidal properties of Chara after observing the absence of larvae in waters where the algae bloomed. These observations were subsequently confirmed and extended by other investigators (Vasconcelos, 1923; Buhot, 1927). More recently, the potential use of Chara in mosquito control was investigated by Amonkar (1969). By utilizing organic extracts from Chara, Amonkar reported impaired mosquito larval development. Utilizing oospores from Chara in nutrient agar, Harrington (1969) showed that the growth of various types of bacteria could also be inhibited. The broad base inhibitory action of Chara isolates was further demonstrated by Wium-Andersen et al. (1982). They showed that two sulfur compounds isolated from C. globularis exhibited allelopathic effects on phytoplankton by inhibiting photosynthesis in the diatom Nitzschia palea. These inhibitory effects have also
been extended to mammalian systems. Su and Staba (1973) reported the growth of amelanoma tumors was delayed in hamsters following intramuscular injection of organic extracts from Chara vulgaris. Similarly, Sherif (1980) observed remission of polyoma tumors in rats after a single intratumor injection of steam distillates from Chara globularis. He also reported an increase in agglutination of polyoma transformed, embryonic rat heart cells, following treatment with Chara. More recently, studies by Gallo (personal communication) showed that steam distillates from Chara globularis were capable of inhibiting the growth of Jensen sarcoma tumors in Sprague-Dawley rats following intratumor injection. Along similar lines, Inman (1986), utilizing organic extracts from steam distillates from Chara globularis, observed a delay in growth of Jensen sarcoma and rat kidney cells in vitro. Additional studies by Gallo also showed that such distillates were able to retard the growth of several neoplastic cell lines in vitro, including Jensen sarcoma, human pancreas epithelioid carcinoma, and human glioblastoma. In contrast, similar treatment of normal human lung cells had no apparent growth retardation or toxic side effects.

It is unlikely that the observed anti-neoplastic action of Chara extracts is primarily attributable to toxic side effects. In vivo studies with rats (Sherif, 1980) and mice (Gallo, personal communication) have indicated that steam distillates from Chara globularis are non-toxic to these animals. For example, distillates given to mice as a substitute for water for a period of three months resulted in no animal deaths attributable to the extract. Su and Staba (1973) assessed possible coagulant or anticoagulant properties of an organic extract from Chara vulgaris.
They reported no changes in prothrombin time or partial thromboplastin time following intraperitoneal injections of mice with the extract.

Only limited information is available on the identity of compounds present in extracts from Chara globularis (Harrington and Gallo, 1985; Anthoni et al., 1980). Two classes of agents present in the mixture, undecanal and various sulfur drugs are known to have anti-neoplastic activities (Higaashikaze, 1981). Undecanal compounds have been shown to exhibit anti-neoplastic activities in SV40 virus-induced tumors in mice (Higashikaze, 1981), while sulfur compounds reportedly exhibit anti-neoplastic activities in vivo and in vitro (Bourgougnon et al., 1994; Hensel, 1995).

Functional Organization of the Cell Nucleus

In an effort to better document the potential anti-neoplastic properties of Chara, the present investigation placed particular emphasis on large-scale nuclear changes in cells exposed to extracts from this alga. What follows is a review of the major structural and functional aspect of nuclear organization.

A. Overview of Nuclear Organization

Structurally the interphase nucleus can be divided into several domains with specific functions (For Review: De Jong et al., 1996; Spector, 1993; Brasch and Ochs 1992) which are shown in figure 1. The most prominent nuclear domains include the nuclear envelope, chromatin, nucleoli, and the interchromatin space. The nuclear envelope helps to segregate, both temporally and spatially, major nuclear processes from those in the cytoplasm.
Figure 1. Diagram illustrating the major components of a mammalian interphase nucleus. The nucleus is separated from the cytoplasm by a nuclear envelope (NE), through which proteins and nuclear transcripts are exported and/or imported via nuclear pores (NP). The most prominent structure in the nucleus is the nucleolus (Nuc) where rRNA genes are located and is the site for rRNA transcription. Condensed chromatin (Ch) is located primarily on the internal periphery of the nucleus, with transcribed or potentially transcribed chromatin looped internally. An array of structures can be found within the interchromatin domain, including perichromatin granules (PG) and fibrils (PF), as well as interchromatin granules (IG), small nuclear bodies (SNB) and coiled bodies (CB).
The flow of molecules between the nucleus and the cytoplasm is regulated via nuclear envelope pores. For example, ribosomal subunits and mRNA molecules must exit this way before coming together in the cytoplasm for protein synthesis. The pores also regulate the entrance of proteins and other molecules into the nucleus for structural purposes and to modulate gene expression and related functions.

Chromatin can be grouped into two distinct domains, euchromatin and heterochromatin. Euchromatin represents chromosomal regions which extend into the interchromatin space and contains genes which are being, or are ready to be, transcribed. Heterochromatin or condensed chromatin represents regions not poised for transcription at that point in time, as well as telomeric and centromeric regions that are linked to the nuclear envelope. All chromosomal regions, however, decondense at some point in time during S-phase when they replicate.

The nucleolus usually appears as a dominant, condensed structure within the nucleus during interphase. This structure can be divided into three main elements: fibrillar centers, dense fibrillar component, and the granular component. The nucleolus is composed of various proteins, like fibrillarin, silver staining nucleolar organizing regions and specific chromosomal regions containing several tandem repeats of genes (rDNA) coding for ribosomal RNA (rRNA).

The interchromatin space is pervaded by an interconnected network of proteins and other macromolecules that may constitute a scaffold or matrix for transcription and replication. Some of these components include interchromatin granules, which are small nuclear ribonucleoprotein (snRNP) clusters, and perichromatin granules and fibrils which are usually located at the
periphery of chromatin, and which constitute newly transcribed pre-mRNA complexes.

Lastly, this nuclear region also contains prominent structures collectively termed nuclear bodies. Nuclear bodies contain a number of well defined molecular components (see below) whose collective functions in these structures remain unclear (For Review: Lamond and Carmo-Fonseca, 1993; Brasch and Ochs, 1992).

B. Nuclear Bodies and Coiled Bodies

Nuclear bodies were first observed by Cajal (1903) in neuronal cells after silver impregnation. He referred to them as "accessory bodies" due to their proximity to nucleoli. Although several different morphotypes were later described through electron microscopy (Bouteille et al., 1982), as a group these structures appear highly conserved in evolution, being found in both plants and animals (Moreno Diaz de la Espina et al., 1982; Raska et al., 1991). Nuclear bodies of all types seem most prevalent in hyperactive cells such as cancerous tissues (Bouteille et al., 1982), virally transformed cells (Jiang et al., 1991) lectin-stimulated lymphocytes (Chaly et al., 1983), and hormonally stimulated tissues (Vagner-Capodano et al., 1982; Brasch and Peters, 1985; and Brasch et al., 1989). Stimulation of such tissues generally induces an increase in the number of nuclear bodies, while absence of stimulation is followed by a decline in frequency.

Recently the designation "coiled bodies" (CBs) has been used to describe the best characterized subclass of nuclear bodies (For Review: Brasch and Ochs 1992; Lamond and Carmo Fonseca, 1993). The formation of CBs appear to come about through nucleolar budding (Dupuy-Coiln and Bouteille, 1972; Vagner-Capodano et al., 1980; Raska et al., 1990), with some
CBs being identified within the nucleolus of some cell lines (Ochs et al., 1994).

The molecular composition of CBs was largely unknown until the discovery of autoantibodies that specifically react with nuclear antigens (For Review: Raska et al., 1991). It is now known that CBs contain several specific molecular components. P80-coilin is an 80Kd molecular weight protein which is prevalent in all coiled body-like structures so far examined (Andrade et al., 1991). The antigen appears in two states, an insoluble state, which forms CBs and a soluble state, which exists both in the nucleus and the cytoplasm. This distribution appears to be cell-cycle dependent (Andrade et al., 1993), and is possibly regulated by hyperphosphorylation of p80-coilin during mitosis (Carmo-Fonseca et al., 1993).

The presence of fibrillarin and silver staining nucleolar organizer proteins in both nucleoli and CBs suggests a functional relationship may exist between these two structures (Raska et al., 1990). Fibrillarin is a 34 KD nucleolar protein, associated with U3 snRNP and fibrillar regions of the nucleolus, which has been identified in some CBs but not all (Ochs et al., 1985; and Ochs and Smetana 1991). The association of fibrillarin with CBs appears to be greater in non-cycling cells relative to cycling cells (Raska et al., 1990).

Another group of important proteins detected in CBs are those associated with pre-mRNA splicing processes, called snRNP's (Raska et al., 1991; Lamond and Carmo-Fonesca, 1993). They are ubiquitous in active cell nuclei and usually appear clustered in interchromatin granules and perichromatin fibrils (Fakan et al., 1984). Differences in snRNP distribution in nuclei from primary cell lines and immortalized cells have been observed (Spector et al., 1992).
Immunofluorescence staining for snRNP's in primary cell lines, exhibited a broadly speckled distribution, along with a small percentage of cell (2-3%) with discrete foci which colocalized with CBs. In contrast, immortal REF-52 cells exhibited a higher percentage (4-40%) of colocalized CB foci, while adenovirus transformed REF-52 cells exhibited an even higher percentage.

The above results confirmed and extended the long-standing observation that cancer and transformed cells contain elevated number of CBs relative to other types of tissues. The localization of snRNP's in CBs appears to be transcription dependent and sensitive to heat shock, actinomycin-D, and α-amanitin, which appears to displace snRNP's from coiled bodies and appears to result in increased association with fibrillarin (Raska et al., 1990; Carmo-Fonseca et al., 1992). Along with snRNP's, snRNA's have also been detected in CBs by in situ hybridization.

In addition to the above components, several other proteins have been detected in coiled bodies. These include DNA topoisomerase I, M3G-capped RNA (Andrade et al, 1991; Raska et al.,1991) and a protein non-snRNP splicing factor, U2AF, which is an auxiliary factor for U2 snRNP (Zamore and Green, 1991). It is likely that as more specific antibodies become available, additional CB-associated components will also be revealed.

Despite containing numerous important nuclear proteins, the precise function of coiled bodies is still unclear (Brasch and Ochs, 1992). However, the association of CBs with nucleoli in both cycling and non-cycling cells, suggests that these bodies may play different functions depending on the physiological state of the cell (Raska et al., 1990). For instance, the presence of various snRNP splicing factors and other agents involved in RNA processing, and the presence of fibrillarin and nucleolar organizing regions, implies that
CBs may play a role in both pre-mRNA processing and/or nucleolus associated processes. Regardless of function, it is clear that CBs are structural indicators of transcriptional activity in nuclei where they are abundant. This suggests that CBs might also be used to monitor the effects of anti-neoplastic agents.

C. Proliferating Cell Nuclear Antigen (PCNA)

The use of antibodies that detect a 36 Kd nuclear auxiliary protein for DNA polymerase delta, known as proliferating cell nuclear antigen (PCNA), can provide further information on the replicative status of cells and tissues (Celis et al., 1984; Dietrich, 1993). PCNA is synthesized in normally proliferating and transformed cells, but is present in only very low amounts in quiescent cells. Like p80-coilin, PCNA also appears to exist in two states, a soluble nucleoplasmic form and an insoluble form which is associated with DNA replication sites. Cells entering late G1 and early S-phase exhibit a immunofluorescence pattern absent in nucleolar labeling. As DNA replication proceeds, PCNA becomes associated with specific nuclear structures and exhibits a granular labeling pattern which appears to coincide with DNA replication sites (Bravo and Macdonald-Bravo, 1987). Completion of DNA synthesis results in a more general staining pattern (Bravo and Macdonald-Bravo, 1985; and Bravo, 1986). PCNA has also been implicated in DNA excision repair. Cells exposed to UV irradiation exhibit granular labeling which coincides with sites of repair (Celis and Madison, 1986; Toschi and Bravo, 1988). Therefore, PCNA may be used as a indicator of the proliferation status of cells and may provide potential information on the mechanism of action for anti-neoplastic agents.
METHODS

Project Overview

A. Cell Lines Characterization

All experimentation for this project was planned using the cancerous cell line, Jensen sarcoma (passage number \( \sim 100 \)), and two non-cancerous lines, Chang liver (passage number \( \sim 300 \)) and (embryonic) Intestine 407 (passage number \( \sim 300 \)). Jensen sarcoma was shown previously to be sensitive to extracts from Chara, both in vivo and in vitro (see above). The cells exhibit contact inhibition in vitro, and are attachment dependent, meaning that they require a solid substrate to undergo cell division (Beltz, 1983). Chang liver and Intestine 407 are immortalized cells by way of continual passage. It is currently unknown whether this immortalization is due to an accumulation of genetic changes in cultures or if variants initially present in the culture were selected for by serial passage.

B. Doxorubicin

Doxorubicin (Adriamycin), a well characterized antibiotic used in treatment of cancers, was chosen as a comparison agent for the Chara extract. Doxorubicin is a broad spectrum antibiotic isolated from a strain of Streptomyces peucetius (Acromone et al., 1969). It works by intercalation between base pairs in the major groove in DNA (Zunino et al., 1972). This intercalation inhibits the enzymes involved in DNA replication and transcription (Bachur et al., 1992; Chuang and Chuang, 1979). Doxorubicin has been observed to exhibit growth inhibitory activity on Chinese hamster ovary cells at the microgram level with short
exposure times as little as 1 hour (Barranco and Novak, 1974). With longer exposure times (24 or more hours) effects have been observed at the nanogram level (Bempong and Brooks, 1977).

C. Experimental Design and Goals

This study examines the overall question, does Chara extract alter specific nuclear indicators, and if so what might this indicate about the possible mechanism(s) of action? Based off of this question, the hypothesis that Chara modulates the appearance of nuclear growth indicators related to transcription and cell-cycle in Jensen sarcoma but not in non-cancerous cells was derived. To address this question, several initial questions were raised and appropriate experiments developed. First, does Chara extract naturally contain possible anti-neoplastic agents? This question was addressed by performing dose response curves in Jensen sarcoma with Chara extract or doxorubicin. A dose which produced an approximate 50% reduction in cell numbers in Jensen sarcoma, as assessed by hemocytometer counts, was chosen. The doses employed in this study were obtained from dose response curves using various concentrations of Chara extract or doxorubicin (data not shown). Based on these initial studies, 5% v/v Chara extract and 5nM doxorubicin were chosen. 2% Chara extract or 2.5nM doxorubicin produced minimal reduction (~10%) in Jensen sarcoma. A 75% reduction of cell density was obtained with 10% Chara extract, while 10nM doxorubicin resulted in no cellular proliferation. The other cell lines were exposed to the same concentration of doxorubicin and/or Chara extract chosen.

Once this was established, the question was raised, does Chara extract alter specific nuclear indicators related to transcriptional activity, and how does this effect compare to
doxorubicin? This question was addressed through immunofluorescence microscopy using antibodies that recognize p80-coilin and fibrillarin. Anti-p80-coilin was used to identify and quantify coiled bodies through fluorescence microscopy. Similarly, anti-fibrillarin was used to quantify nucleoli and small peripheral structures. Anti-fibrillarin antibodies were also used to differentiate between fibrillarin positive and negative labeled CBs. Since CBs can serve as indicators of nuclear and nucleolar hyperactivity (Bouteille et al., 1987; Brasch and Ochs, 1992), changes in their numbers can be used as broad indicators of both transcriptional levels and nucleolar function.

These microscopic studies were accompanied by western blot quantification of SDS gel-separated cellular proteins to determine total cellular amounts of p80-coilin.

Finally, the question was asked, does Chara extract alter the cycle-cycle, and how does this effect compare to doxorubicin? This question was addressed by employing immunofluorescence microscopy using anti-PCNA antibodies. The PCNA labeling pattern was used to score the phases of cell division. PCNA is a nuclear protein correlated with proliferation of cells during the S-phase and becomes associated with replication sites (Bravo and Macondo-Bravo, 1987).

This study provides data on the effects of Chara extract on the growth of cancerous and both non-cancerous cell lines, utilizing some very specific nuclear components as indicators and then by comparing these effects to those of doxorubicin, a well defined anti-neoplastic agent. In addition to the questions mentioned above, several other questions are addressed. 1) Does the average number of CBs vary between the neoplastic cells and the non-cancerous lines, and if so what might this difference mean? 2) What changes occur in CBs in the three cell lines during proliferation? 3) If there is a
change, what could this indicate regarding transcriptional activity in relation to the growth of individual cell lines?  
4) If fibrillarin and p80-coilin reveal two different populations of CBs, is there a change in these populations during proliferation?  
5) Does PCNA, which is an indicator of cellular proliferation, differ among the three cell lines, does it vary with respect to the age of the culture, and if so, what might this indicate?

The cell enumeration data shown in this study was derived from 3 independent experiments employing 3 separate batches of Chara extract and doxorubicin to monitor activity changes between batches. To control for possible batch variations, the same batch of Chara extract and doxorubicin was used in all three cell lines and includes cell enumeration and all subsequent experiments. Additionally, all the experiments for the three cell lines were set up during the same period. Due to the variability of these nuclear indicators in asynchronous cultures, at least a 100 cells from each cell, treatment and time period were enumerated.

D. Experimental Analysis

All cell growth data, CB, fibrillarin labeled structure counts, and nuclear PCNA pattern quantitation were arranged in an a x b factorial design and subjected to analysis of variance (ANOVA) (Milton and Tsokos, 1985; Dowdy and Wearden, 1985) using SuperAnova software (Abacuss). Data sets exhibiting significant interaction were assessed for the influence of each factor independently. Pairwise comparisons were made with treatment (control, Chara and doxorubicin) and time (24, 48, 72, 96, 120... hrs.) as factors using Duncan's multiple range test with Kramer's adjustment for unequal sample sizes (Kramer, 1956). Comparisons between the cell
types were made within the control groups with cell type (Jensen sarcoma, Chang liver and Intestine 407) and time as factors. To meet the assumption of homogeneous variances (homoscedasticity), the sample variances were subjected to Bartlet's test. Experiment sets exhibiting significant heterogenous variances were transformed using the equations \( y = \log e \) for cell enumeration and \( y = \log e + 10 \) for p80-coilin, fibrillarin enumeration and all data expressed as a percentage. Results expressed as significant implies \( P < 0.05 \) unless otherwise stated. All PCNA data was expressed as weighted mean ± SE. The remaining data was expressed as mean ± SE.

Procedures

A. Cell Cultures

Except where indicated, all basic techniques involving cell culturing follow general procedures outlined in Freshney (1983). Cultures were maintained in media optimized for each respective cell line as outlined by American Tissue Culture Collection (ATCC). Jensen sarcoma cells (ATCC CCL45) were grown in McCoy's 5A media at pH 7.4 and 5% fetal bovine serum (FBS). Chang Liver cells (ATCC CCL13) and Intestine 407 cells (ATCC CCL6) were cultured in Basal modified Eagle's (BME) media with Earle's salt solution at pH 7.4 with 10% (FBS) and 15% (FBS) respectively. All cultures were supplemented with 1% antibiotic-antimycotic solution containing 10,000 U/ml penicillin-G, 10,000 \( \mu \)g/ml streptomycin sulphate, and 25 \( \mu \)g/ml of amphotericin B. Culture media was buffered with 10mM sodium bicarbonate. All cultures were maintained in a VWR Scientific model 2100 humidified incubator at 37°C with 5% CO2 - 95% air flow. Stock cultures, maintained in 25 cm\(^3\) or 75 cm\(^3\) flat-bottomed
polystyrene culture flasks were subcultured prior to confluency to maintain cells in log-phase.

To remove cells from the culture flask, they were treated with 0.25% trypsin and 1mM EDTA at 37°C for 5 minutes, followed by addition of fresh media and centrifugation at 2,000g for 5 minutes. Cell pellets were washed in media with FBS and centrifuged again, followed by resuspension in fresh culture media. Fresh culture flasks were then inoculated with cell suspension. Experimental cultures were established in similar manner and then allowed 24 hrs to recover from trypsin treatment before exposure to Chara extracts or doxorubicin (Barranco et al., 1980). All cultures were replenished with fresh culture media every 48 hrs following treatment.

All cultures were grown in Corning plates or flasks except where otherwise indicated. Culture reagents were obtained from Gibco Laboratories, Grand Island, N.Y.. Culture manipulations were performed in a Labconco class II vertical flow hood.

B. Preparation of Anti-neoplastic Agents

Extracts were prepared from the fresh water alga Chara globularis Thuillier, grown in a special retaining tank on the CSUSB campus. After harvesting, the material was washed thoroughly with double-distilled water and then blotted dry. 200-400 grams of material was then placed in a stainless steel juice extractor, and 50-80 ml of eluent was collected. The eluent was sterile filtered (0.2μm acetate filter) and frozen in 5 ml aliquots at -80°C. Doxorubicin-HCl (Sigma) solution was prepared by suspending 10mg in 10 mL of sterile filtered double-distilled water (0.2 μm acetate filters). Aliquots were stored at -80°C. A 100μg/ml stock solution was prepared by serial dilution and stored at 4°C.
C. Preparation of Culture Media

Culture media was prepared just prior to use in the following manner. Thawed Chara extract was sterile filtered (0.2µm acetate filter) to remove precipitated material which could contribute to background autofluorescence. An appropriate volume of extract or doxorubicin solution was added to sterile filtered (0.2 µm acetate filter) incomplete media, to achieve 5% v/v Chara extract or 5nM Doxorubicin. The addition of FBS and antibiotic resulted in final desired concentration of all media components. Control media was prepared with sterile water.

D. Cellular Proliferation

An inoculum of 2ml containing 4 X 10^4 cells/ml of Jensen sarcoma or 5 X 10^4 cells/ml of non-cancerous cell lines was applied per well in 6-well (34.6mm diameter) polystyrene culture plates. Cells were harvested every 24 hrs after plating by exposure to 0.25% trypsin and 1mM EDTA at 37°C for about 5 min, followed by centrifugation at 2,000g for 5 min. Cell pellets were resuspended in 1 ml of media containing 0.4% trypan blue (Sigma). The number of viable cells were then counted using a hemocytometer. Additionally, cells were enumerated by visual inspection within the cell cultures. This was done by adding trypan blue to cultures and counting the cells under a Nikon inverted microscope using an ocular grid calibrated using a micrometer.

E. Immunological Techniques

Except where indicated, all immunological techniques were based on methods outlined in The American Society for Cell Biology Education Committee Workshop (1992). Nunc 4-
chamber tissue culture slides were inoculated with 0.7 ml per chamber of previously mentioned cell densities. Sample slides were taken at 24 hr intervals after treatment for the duration of each experiment.

Slides were washed with 10mM phosphate buffered saline (PBS) at pH 7.4, and cells were fixed with 2% EM-grade formaldehyde/PBS for 20 min at room temperature (RT). After washing 3 X 10 min with PBS, the sample slides were stored at 4°C in PBS. Cells were permeabilized with 0.25% Triton X-100 for 5 min at RT followed by a PBS wash. The slides were then incubated for 1 hr at 37°C in a humidified chamber, with 50µl human antibody (AK) to PCNA (Andrade et al., 1993) or double-labeled with rabbit antibody (R288) to p80 coilin (Andrade et al., 1993) and mouse monoclonal antibody (72B9) to fibrillarin (Reimer et al., 1987) at 1:200 dilution. All primary antibodies were generously donated by Dr. Robert L. Ochs, W.M. Keck Autoimmune Disease Center, The Scripps Research Institute, La Jolla, CA. Slides were washed 3 X 10 min with PBS at room temperature (RT) and rinsed prior to addition of 200µL of appropriate secondary antibodies linked to FITC or rhodamine (Vector,). Samples were incubated under the same conditions as the primary antibodies. Slides were then again washed with PBS followed by double-distilled water. Coverslips were mounted using Fluorsave (Calbiochemical, La Jolla, CA) to minimize quenching of the fluorescence signal.

Specimens were examined under 400X with a Nikon Optiphot microscope equipped with phase contrast optics and an episcopic-fluorescence attachment EF-D containing excitation filters B2A at 495nM for FITC and G2A at 546nM for rhodamine. Cells were photographed with a UFX-II camera system using Ektachrome 400 or Fujichrome 100 slidefilm. Coiled bodies were quantified directly from 35mm slides examined at constant magnification in a slide projector. PCNA stained
nuclei were scored on the basis of immunofluorescence patterns as outlined in Bravo (1986) and Bravo and MacDonald-Bravo (1985).

F. Western Blotting

Samples were prepared by inoculating 25 cm² culture flasks with 5 mL of cell suspension. After plating, cells were harvested every 48 hrs by treatment with 0.25% trypsin and 1 mM EDTA at 37°C for 5 min. Culture media and FBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) was then added and the preparation was centrifuged 2,000g for 5 min. Cell pellets were resuspended and centrifuged in culture media containing 1 mM PMSF, twice. Each pellet was resuspended with an equal volume of PBS and treatment buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) to attain a concentration of 2 X 10⁴ cells/µL as determined from hemocytometer counts. Molecular weight markers were treated the same way.

Cells were subsequently lysed by drawing the sample in and out of a Hamilton syringe prior to heating at 95°C for 5 min followed by storage at -20°C. Samples were thawed and heated at 95°C for 5 minutes prior to loading 10 µL of sample on a discontinuous polyacrylamide gel containing 0.1% SDS (Laemmli, 1970), with the following proteins for molecular weight comparison: bovine albumin (66Kd), egg albumin (45Kd), glyceraldehyde 3-phosphate dehydrogenase (36Kd), and carbonic anhydrase (29Kd). A 5% stacking gel and 10% separating gel were used in a Bio-Rad mini-protein II vertical gel apparatus. Electrophoresis was performed with a 0.025 M tris, 0.192 M glycine and 0.1% SDS pH 8.3 buffer at 15 mA, set to constant current for 90 min at room temperature (RT) using a Savant Instrument Inc. power supply model CVR300.
After electrophoresis, the gels and 0.2μm nitrocellulose paper (S&S NC) were prepared for electrotransfer by soaking in Towbin transfer buffer for 30 min. Proteins were transferred to nitrocellulose membranes in a Bio-Rad mini-trans blot system at 50V/gel under constant current for 2 hr at 4°C. The transfer buffer was continuously mixed with a magnetic stirrer.

Following protein transfer the nitrocellulose membranes were stained with 0.5% Ponceau S stain for 30 seconds and rinsed in water to determine the eveness of the transfer. The nitrocellulose membranes were destained by rinsing in PBS. The gels were stained 3 hr with 0.125% coomassie blue R-250, 50% methanol, 10% acetic acid and destained 1 hr with 50% methanol, 10% acetic acid followed by 7% acetic acid and 5% methanol until the background was clear. Nitrocellulose membranes were prepared for immunoblotting by blocking membranes with 5% non-fat milk in 100mM tris buffered saline (M-TBS) at pH 7.5 for 1 hr at RT. Membranes were incubated for 1 hr at 1/200 dilution in M-TBS of human antibody Shiptak against p80-coilin with gentle aggitation. Membranes were washed 3 X 30 minutes in M-TBS, then incubated with biotinylated anti-human antibody at 1:1000 dilution in M-TBS for 1 hr at RT. Membranes were then washed 3 X 10 minutes in tris buffered saline at pH 7.5 (TBS) followed by incubation of avidin-HRP at 1:1000 dilution in TBS for 1 hour. After washing 3 X 10 minutes in TBS, membranes were colorized with 4-chloro-1-napthol followed by rinsing in TBS followed by double-distilled water. Immunoblots were digitized and relative optical densities obtained using an IS 1000 Digital Imaging system (Alpha Innotech Corporation) at Loma Linda University.
RESULTS

Preparation of Chara Extract

Previous methods for the preparation of a Chara extract involved collection of steam distillates derived from the alga. For this study, a new method was employed for the preparation of the extract. This involved direct extraction of soluble agents from the alga using a juice extractor, followed by filtration with 0.2μm acetate filter.

The final extract exhibited a light yellowish/green color. Addition of 5% v/v of the Chara extract did not appear to affect the pH of the culture media, since no apparent change was observed with the phenol red indicator.

Effect of Agents on Cell Proliferation

Prior to treatment with anti-growth agents, the growth kinetics of untreated cell lines were examined to establish a control base line (figure 2). Jensen sarcoma exhibited a lag period of 24 hrs following plating, prior to a noticeable increase in cell density. Other cells lines exhibited noticeable proliferation within the same time period. The cell density of the various lines after 7 days in culture varied significantly. Jensen sarcoma reached a peak density of about 6.5 X 10^6 cells/well, while Intestine 407 and Chang liver attained a density 3.5 X 10^6 cells/well and 2.1 X10^6 cells/well, respectively (figure 2).

The addition of 5nM doxorubicin or 5% v/v Chara extract to the three cell lines markedly affected their growth kinetics (figure 2). With Jensen sarcoma, treatment with Chara extract resulted in a significant decrease in cell density after 24 hr, while doxorubicin produced a significant decrease in cell density, relative to controls, only 48 hrs
after treatment. There were no significant growth differences between Chara extract and doxorubicin treated cells at each time point. Compared to Jensen sarcoma, Chang liver and Intestine 407 responded quite differently to treatment with Chara extract and doxorubicin. First, both cell lines exhibited significant reaction to Chara treatment only in the plateau growth phase, when measurable difference in density were evident between 120 and 144 hrs for Intestine 407 and 96-144 hrs for Chang liver. Interestingly, doxorubicin significantly affected Intestine 407 cells after 24 hrs relative to both controls and Chara treated cultures.

Assessment of the growth kinetics by microscopy, shows a similar behavior as seen with the hemocytometer counts (Figure 3). This method of cell enumeration did, however, result in a lower total cell count per well compared to the hemocytometer method. This could be accounted for by the knowledge that in small cultures, such as 6-well plates, cells cluster at the edges of the wells. If samples are not taken in such areas of the well, the cell density would be underestimated.

Morphological Changes

Jensen sarcoma cells normally exhibited a spindle shaped morphology during interphase (figure 4). However 72 hrs after treatment with either Chara extract or doxorubicin, marked morphological changes were evident. The cells now exhibited hyperplasia along with long cytoplasmic extensions (figure 4). In contrast, the cytoplasmic borders of the non-cancerous cell lines appeared flattened and round. Treatment with either doxorubicin or Chara did not appear to result in an altered morphology (figure 4).
Figure 2. Logarithmic growth curves of Jensen sarcoma, Intestine 407 and Chang liver cells as assessed by trypan blue exclusion following dissociation with trypsin and EDTA. Initial plating densities are indicated at (P) time period, while initial exposure to Chara extract and doxorubicin are indicated by (T). Culture medium was replaced every 48 hrs. Significant differences as assessed by Duncan's multiple range test (P<0.05) are indicated as (#) for untreated versus Chara treated, (*) for doxorubicin treated versus untreated and (+) for Chara versus doxorubicin treated cells. Data represent the mean ± SE of 3 independent experiments performed in triplicate.
Figure 3. Logarithmic growth curves of Jensen sarcoma, Intestine 407 and Chang liver cells as assessed by trypan blue exclusion. Initial plating densities are indicated at (P) time period, while initial exposure to Chara extract and doxorubicin are indicated by (T). Culture medium was replaced every 48 hrs. Significant differences as assessed by Duncan's multiple range test (p<0.05) are indicated as (#) for untreated versus Chara treated, (*) for doxorubicin treated versus untreated and (+) for Chara versus doxorubicin treated cells. Data represent the mean ± SE of 3 separate areas in each well
Figure 4. Phase contrast photomicrographs of control, *Chara* extract and doxorubicin treated cells from Jensen sarcoma, Intestine 407 and Chang liver cells. Magnification 1500X.
Effects of Agents on Nuclear Indicators

As outlined in the introduction, the nuclear protein fibrillarin is present in nucleoli, certain peri-nucleolar structures and coiled bodies (CBs). However, preliminary studies by Brasch et. al., (1994), using double labeling with mouse monoclonal antibody (72B9) to fibrillarin and rabbit antibody (R288) to p80-coilin, revealed significantly less than 100% coincident labeling of CBs by the two antibodies. Although all CBs appeared p80-coilin positive, not all were also fibrillarin positive, suggesting that two overlapping populations were present, at least operationally.

To investigate this possibility further and to avoid ambiguity in identifying and quantifying fibrillarin and coilin-containing structures, data from each antibody were first analyzed independently.

A. Fibrillarin

Immunofluorescence localization of fibrillarin-containing structures in Jensen sarcoma, Intestine 407 and Chang liver cells by the anti-fibrillarin antibody 72B9 is shown in figure 5. Comparison of phase contrast and fluorescent images of the same cells (data not shown) revealed that the most prominent labeled regions coincided with nucleoli while CBs and other smaller labeled structures appeared throughout the interchromatin domain.

This pattern of fibrillarin-specific labeling has been widely reported in many other cells and tissues and is thought to represent primarily pre-nucleolar bodies and elements associated with RNA processing (Spector, 1993). Despite the relatively small fluctuations with respect to treatment and time (figure 6 and table 1), fibrillarin
Figure 5. Photomicrographs of control, *Chara* extract and
doxorubicin treated cells from Jensen sarcoma, Intestine 407
and Chang liver cells labeled with mouse monoclonal antibody
72B9 against the nuclear protein fibrillarin. Nuclear
structures labeled with anti-fibrillarin antibody are
indicated by arrows. Magnification 1500X.
Figure 6. Quantitation of fibrillarin containing nuclear structures in Jensen sarcoma, Intestine 407 and Chang liver. Bars with an (*) above indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the mean ± SE of 100-250 cells.
Average Number of Fibrillarin Labeled Structures/Nucleus

Chang liver Intestine 407 Jensen sarcoma

**CONTROL**  **CHARA**  **DOX**
Table 1. Pairwise comparison of the influence of time on the average number of fibrillarin labeled structures for each cell type within control, Chara and doxorubicin.

<table>
<thead>
<tr>
<th>Group (Hrs)</th>
<th>Time</th>
<th>Jensen Sarcoma</th>
<th>Intestine 407</th>
<th>Chang liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con 24</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>S S S S</td>
<td>S NS NS NS  NS</td>
<td>NS S NS NS</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>- NS NS NS</td>
<td>- NS S S  NS</td>
<td>- S NS NS</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>- - NS NS</td>
<td>- - NS S  S</td>
<td>- - S S</td>
</tr>
<tr>
<td></td>
<td>Chara 24</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>S NS S S</td>
<td>S S S NS</td>
<td>S S S NS</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>- NS NS S</td>
<td>- NS NS S</td>
<td>- NS S S</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>- - NS NS</td>
<td>- - NS S</td>
<td>- - NS S</td>
</tr>
<tr>
<td></td>
<td>Doxo 24</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>S S S S</td>
<td>S NS NS S</td>
<td>S S S NS</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>- NS S NS</td>
<td>- NS S NS</td>
<td>- NS S NS</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>- - NS NS</td>
<td>- - NS NS</td>
<td>- - NS NS</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
distribution patterns were surprisingly uniform and similar in all three cell types. Nuclei in control Jensen sarcoma cells, typically exhibited one (or more) large fibrillarin-rich nucleolar regions as well as smaller peripheral structures and usually one distinct CB adjacent to the nucleolus or within the interchromatin region. Similar fibrillarin distribution was evident in Chara and doxorubicin treated Jensen sarcoma, however the periphery of the nucleolus appeared less irregular (figure 5). In the non-cancerous cells, fibrillarin containing structures appeared smaller and more numerous than those observed in Jensen sarcoma (figure 5 and 6).

B. Coiled Bodies

As outlined in the introduction, in addition to several molecules associated with pre-mRNA and rRNA processing, coiled bodies are characterized by at least one unique protein, p80-coilin. Immunofluorescence localization of CBs by the anti-p80-coilin antibody R288 in Jensen sarcoma, Intestine 407 and Chang liver is shown in figure 7. Quantitative analysis of CBs identified by R288 revealed marked changes, not only with respect to cell type, but also in relation to time and treatment regime (figure 8 and table 2). This was particularly dramatic in Jensen sarcoma.

Initially at 24 hrs, under all treatment conditions, an average of 0.19 ± 0.05 or less CBs per nucleus was observed in Jensen sarcoma cells. In control cells this value rose progressively thereafter and by 72 hrs effectively all nuclei contained at least 0.96 ± 0.08 CB on average. This was followed by a gradual decline and at confluency, an average of 0.67 ± 0.07 CBs per nucleus was the norm.

A significantly different pattern emerged in Jensen sarcoma treated with Chara extract or doxorubicin.
Figure 7. Photomicrographs of control, Chara extract and doxorubicin treated cells from Jensen sarcoma, Intestine 407 and Chang liver cells labeled with rabbit antibody R288 against the protein p80-coilin. p80-coilin labeled structures are indicated by arrows. Magnification 1500X.
Jensen Sarcoma

Chang liver

Intestine 407

Control | Chara | Doxorubicin
Figure 8. Quantitation of p80-coilin containing coiled bodies in Jensen sarcoma, Intestine 407 and Chang liver. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the mean ± SE of 100-250 cells.
Jensen sarcoma

Intestine 407

Chang Liver

Avg CB/Cell

Time (Hr)

CONTROL

CHARA

DOX

Avg CB/Cell

Avg CB/Cell

Avg CB/Cell

24 48 72 96 120
Table 2. Pairwise comparison of the influence of time on the average number of p80-coilin containing structures for each cell type within control, Chara and doxorubicin.

<table>
<thead>
<tr>
<th>Group (Hrs)</th>
<th>Jensen sarcoma</th>
<th>Intestine 407</th>
<th>Chang liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
</tr>
<tr>
<td>Con 24</td>
<td>S S S S</td>
<td>S NS NS S</td>
<td>NS NS S S</td>
</tr>
<tr>
<td>Con 48</td>
<td>- NS NS NS</td>
<td>- NS NS NS</td>
<td>- NS NS S</td>
</tr>
<tr>
<td>Con 96</td>
<td>- - NS NS</td>
<td>- - NS S</td>
<td>- - NS S</td>
</tr>
<tr>
<td>Chara 24</td>
<td>S S S S</td>
<td>NS S S NS</td>
<td>S S S S</td>
</tr>
<tr>
<td>Chara 48</td>
<td>- NS NS NS</td>
<td>- S S NS</td>
<td>- NS S S</td>
</tr>
<tr>
<td>Chara 96</td>
<td>- - NS NS</td>
<td>- - NS S</td>
<td>- - S S</td>
</tr>
<tr>
<td>Doxo 24</td>
<td>NS NS S S NS</td>
<td>NS S S S</td>
<td>- - NS S</td>
</tr>
<tr>
<td>Doxo 48</td>
<td>- NS S S NS</td>
<td>- S S S</td>
<td>- - NS S</td>
</tr>
<tr>
<td>Doxo 96</td>
<td>- - S S</td>
<td>- - NS S</td>
<td>- - S</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
Both agents had a significant inhibitory effect on CB development, which was particularly strong with doxorubicin. With both agents, the average number of CBs never exceeded 0.57 ± 0.06 and indeed with doxorubicin, never exceeded 0.41 ± 0.07 CBs per nuclei.

A significantly more mixed pattern of CB development was evident in Intestine 407 cells (figure 8). Under control conditions, the average fluctuated between 1.41 ± 0.08 and 2.11 ± 0.10 CBs per nucleus for the duration of the culture. The presence of Chara extract or doxorubicin did have a generally negative effect on CBs average over time, but this appeared far more varied than in Jensen sarcoma (figure 8 and table 2).

Control Chang liver cells averaged the largest number of CBs per nucleus of all three cell lines ranging around 2.45 ± 0.12 to 3.21 ± 0.16 CBs per nuclei (figure 8). A slow but significant decrease in these values was evident over time, an effect that was enhanced in the presence of Chara extract. Remarkably, however, even at confluence the number of CBs still averaged around 2 in this cell line.

C. Coiled Body Subpopulation

As previously mentioned, although all identifiable CBs were labeled by antibodies against p80-coilin, not all those bodies were recognized by the anti-fibrillarin antibody 72B9. Quantitative analysis of these two CB populations revealed major differences with respect to cell type, time and antineoplastic treatment (figure 9,10,11 and tables 3,4,5).

In Jensen sarcoma, the average number of fibrillarin positive and negative CBs at 24 hrs was less than 0.2/nucleus under all treatment conditions. In control Jensen sarcoma the number of fibrillarin positive CBs rose significantly thereafter peaking at 72 hrs with a gradual decline at
Figure 9. Quantitation of coiled body subpopulation in Jensen sarcoma. Fibrillarin positive CBs, fibrillarin negative CBs and percentage of total CBs positive for fibrillarin. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the mean ± SE of 100-250 cells.
Table 3. Pairwise comparison of the influence of time in Jensen sarcoma within control, Chara extract and doxorubicin treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs.

<table>
<thead>
<tr>
<th>Group (Hrs)</th>
<th>Time</th>
<th>Fibrillarin Positive CBs</th>
<th>Fibrillarin Negative CBs</th>
<th>% Total Fibrillarin Positive CBs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
<td>48 72 96 120</td>
</tr>
<tr>
<td>Con 24</td>
<td>S</td>
<td>S  S  S  S</td>
<td>S  S  S  S</td>
<td>NS  NS  NS  NS</td>
</tr>
<tr>
<td>Con 48</td>
<td>-</td>
<td>-  S  S  NS</td>
<td>-  NS  NS  NS</td>
<td>-  NS  NS  NS</td>
</tr>
<tr>
<td>Con 72</td>
<td>-</td>
<td>-  NS  S  NS</td>
<td>-  -  NS  NS</td>
<td>-  -  NS  NS</td>
</tr>
<tr>
<td>Con 96</td>
<td>-</td>
<td>-  -  -  S</td>
<td>-  -  -  NS</td>
<td>-  -  -  NS</td>
</tr>
<tr>
<td>Chara 24</td>
<td>S</td>
<td>S  S  S  S</td>
<td>NS  NS  NS  S</td>
<td>NS  NS  NS  NS</td>
</tr>
<tr>
<td>Chara 48</td>
<td>-</td>
<td>-  NS  NS  S</td>
<td>-  NS  NS  S</td>
<td>-  NS  NS  NS</td>
</tr>
<tr>
<td>Chara 72</td>
<td>-</td>
<td>-  NS  NS  NS</td>
<td>-  -  NS  S</td>
<td>-  -  NS  NS</td>
</tr>
<tr>
<td>Chara 96</td>
<td>-</td>
<td>-  -  -  S</td>
<td>-  -  -  S</td>
<td>-  -  -  NS</td>
</tr>
<tr>
<td>Doxo 24</td>
<td>NS</td>
<td>NS  S  NS  S</td>
<td>NS  NS  NS  NS</td>
<td>NS  NS  NS  NS</td>
</tr>
<tr>
<td>Doxo 48</td>
<td>-</td>
<td>-  NS  S  NS</td>
<td>-  NS  NS  NS</td>
<td>-  NS  NS  NS</td>
</tr>
<tr>
<td>Doxo 72</td>
<td>-</td>
<td>-  S  NS  NS</td>
<td>-  -  NS  NS</td>
<td>-  -  NS  NS</td>
</tr>
<tr>
<td>Doxo 96</td>
<td>-</td>
<td>-  -  S  NS</td>
<td>-  -  -  NS</td>
<td>-  -  -  NS</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
Figure 10. Quantitation of coiled body subpopulation in Intestine 407. CBs labeled with fibrillarin, CBs with no labeling for fibrillarin, and percentage of CBs labeled with fibrillarin. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the mean ± SE of 100-250 cells.
Table 4. Pairwise comparison of the influence of time in Intestine 407 within control, Chara extract and doxorubicin treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs.

| Group | Time (Hrs) | 48  | 72  | 96  | 120 | 48  | 72  | 96  | 120 | 48  | 72  | 96  | 120 |
|-------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Con   | 24         | S   | -   | S   | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  |
| Con   | 48         | -   | S   | S   | S   | NS  | NS  | NS  | S   | S   | S   | S   | S   | S   |
| Con   | 72         | -   | NS  | S   | NS  | NS  | NS  | S   | S   | NS  | NS  | S   | S   | S   |
| Con   | 96         | NS  | S   | -   | NS  | S   | NS  | S   | NS  | NS  | NS  | S   | S   | S   |
| Chara | 24         | S   | S   | S   | S   | NS  | S   | S   | NS  | NS  | S   | S   | NS  | NS  |
| Chara | 48         | NS  | NS  | NS  | NS  | S   | S   | NS  | NS  | NS  | S   | S   | NS  | NS  |
| Chara | 72         | -   | NS  | S   | NS  | NS  | NS  | S   | S   | NS  | NS  | S   | S   | NS  |
| Chara | 96         | -   | NS  | -   | NS  | S   | NS  | S   | NS  | NS  | S   | S   | NS  | NS  |
| Doxo  | 24         | S   | S   | S   | S   | S   | S   | S   | NS  | NS  | NS  | NS  | NS  | NS  |
| Doxo  | 48         | NS  | S   | S   | S   | S   | S   | NS  | NS  | NS  | NS  | NS  | NS  | NS  |
| Doxo  | 72         | NS  | NS  | NS  | NS  | S   | S   | NS  | NS  | NS  | NS  | NS  | NS  | NS  |
| Doxo  | 96         | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  | NS  |

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
Figure 11. Quantitation of coiled body subpopulation in Chang liver. CBs labeled with fibrillarin, CBs with no labeling for fibrillarin, and percentage of CBs with fibrillarin labeling. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the mean ± SE of 100-250 cells.
Table 5. Pairwise comparison of the influence of time in Chang liver within control and Chara extract treated cells for number of fibrillarin positive and negative, p80-coilin containing structures along with % total fibrillarin positive CBs.

<table>
<thead>
<tr>
<th>Group (Hrs)</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con 24</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Con 48</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>NS</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Con 72</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>Con 96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chara 24</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chara 48</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>S</td>
<td>-</td>
<td>NS</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>NS</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chara 72</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chara 96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
confluency, while fibrillarin negative CBs remained constant after an increase at 48 hrs (figure 9).

Treatment with either Chara extract or doxorubicin appeared to have inhibitory effects on development in both fibrillarin positive and negative CBs (figure 9). The effects of doxorubicin was particularly strong on both fibrillarin positive and negative CBs, while Chara's inhibitory effects appeared stronger on fibrillarin negative CBs as compared to those labeled with fibrillarin.

Even with the observed inhibitory effects of both Chara and doxorubicin on fibrillarin positive and negative CBs, the relative proportions of these two types of CBs appears to be relatively unchanged compared to control Jensen sarcoma except for a significant increase in fibrillarin positive CB's at 24 hrs treated with doxorubicin (figure 9).

In control Intestine 407 cells (figure 10), the pattern of development for fibrillarin positive CBs was mixed over time, while fibrillarin negative CBs remained fairly constant. Addition of either Chara extract or doxorubicin produced mixed effects on both CB types with respect to time. However, assessment of the relative proportions of fibrillarin positive and negative CBs remains constant between control and doxorubicin treated cells, while Chara treated cells exhibited a higher proportion of fibrillarin positive CBs early on.

For both control and Chara treated Chang Liver (figure 11), the appearance of fibrillarin positive and the proportion of fibrillarin positive CBs appeared to increase with age in culture, while fibrillarin negative CBs appeared to decrease with age in culture. Treatment with Chara extract appeared to have only very modest inhibitory effects on both fibrillarin positive and negative CB development.
D. Western Blot Analysis

P80-coilin has been detected in all vertebrate cells examined so far. In the present study, western blotting analysis of SDS-PAGE separated polypeptides from all 3 cell lines was undertaken for two reasons. First, it was necessary to establish and confirm that p80-coilin was present in these cells. Second, it was necessary to validate independently the presence of this protein with respect to the immunofluorescence analysis of CBs.

The human antibody against p80-coilin (shiptak), identified a single band in western blots of SDS-PAGE separated proteins in all three cell lines (figure 12). Moreover, this single band was evident under all treatments and time points examined as assessed by densitometric analysis (table 6).

The densitometric analysis also suggested that considerable quantitative differences in p80 content were manifest not only among the three cells lines but also under various treatment/growth conditions. This would have to be verified, however, by repeat experiments and more rigorous statistical analysis.

E. PCNA Quantification

As described in the introduction, proliferating cell nuclear antigen (PCNA) can be used as an indicator of the replicative status of cells. In this study three major labeling patterns were observed with human antibody AK against PCNA (figure 13): a) homogeneous labeling of the entire nucleus, b) granular labeling throughout the nucleus, and c) labeling of the nucleus excluding nucleolar regions. These patterns were operationally termed homogenous, granular and nucleolus-negative respectively.
Figure 12. Western blot using the human antibody Shiptak against p80-coilin in Jensen sarcoma, Intestine 407 and Chang liver. Control, *Chara* extract and doxorubicin treated cells were examined, following 24, 72, and 120 hrs after treatment.
Table 6. Relative optical densities in western blot of p80-coilin using the human antibody Shiptak.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (Hrs)</th>
<th>Jensen sarcoma</th>
<th>Intestine 407</th>
<th>Chang liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>24</td>
<td>0.70</td>
<td>0.75</td>
<td>0.92</td>
</tr>
<tr>
<td>CHARA</td>
<td>24</td>
<td>0.73</td>
<td>0.81</td>
<td>0.94</td>
</tr>
<tr>
<td>DOX</td>
<td>24</td>
<td>0.87</td>
<td>0.73</td>
<td>-</td>
</tr>
<tr>
<td>CONTROL</td>
<td>72</td>
<td>0.94</td>
<td>0.77</td>
<td>0.87</td>
</tr>
<tr>
<td>CHARA</td>
<td>72</td>
<td>0.92</td>
<td>0.59</td>
<td>0.96</td>
</tr>
<tr>
<td>DOX</td>
<td>72</td>
<td>0.96</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>CONTROL</td>
<td>120</td>
<td>0.82</td>
<td>0.99</td>
<td>1.00</td>
</tr>
<tr>
<td>CHARA</td>
<td>120</td>
<td>1.00</td>
<td>0.74</td>
<td>0.99</td>
</tr>
<tr>
<td>DOX</td>
<td>120</td>
<td>0.74</td>
<td>0.82</td>
<td>-</td>
</tr>
</tbody>
</table>

Relative O.D. values were derived using the IS 1000 Digital Imaging system by integrating the density area from each band.
Figure 13. Photomicrographs of human antibody AK against PCNA in untreated and treated Jensen sarcoma, Intestine 407 and Chang liver. The various PCNA labeling patterns observed within the cells are denoted by homogenous (a), granular (b) and no nucleolar labeling (c). Magnification at 1500X.
The nucleolus-negative patterns are characteristic of cells in late G1 and entering early S-phase, while granular appearing cells are in active S-phase proceeding to G2 phase and homogenous labeling patterns correspond to the remaining portions of interphase (Bravo and Macdonald-Bravo, 1985).

The assessments of PCNA labeling for all times and treatment conditions in Jensen sarcoma are summarized in figure 14 and table 7. It is noteworthy that, overall, over 50% of the cells under all treatment conditions exhibited nucleolus-negative staining patterns. This corresponds to the late G1 and early S phase transition (Bravo and Macdonald-Bravo, 1985) and remained fairly constant over time. In contrast, granular (active S-phase) and homogenous (G1 and G2 phase) labeling patterns varied significantly in control cultures. At 24 hrs, about 5% of cells showed granular patterns. This rose to over 15% at 72 hrs and gradually declined as the cultures reached confluency. Homogenous labeling was highest (~35%) at 24 hrs and at confluency, with lower values in between.

Unlike Jensen sarcoma, intestine 407 cells exhibited predominantly (50-70%) homogenous (G1 and G2) labeling patterns at all times and under all treatment conditions (figure 15 and table 8). Nucleolar-negative (G1→S) labeling was evident in less than 30% of cells at all times except confluency, when nearly 50% of cells shared that pattern. Relative to both Jensen sarcoma and intestine 407, Chang liver displayed a more even proportion of cells with either homogenous (G1 and G2) or nucleolus-negative (G1→S) labeling under the two treatment conditions examined (figure 16 and table 9). Despite the initial gradual decline in granular labeled cells in Chang liver no statistical differences were observed over time with respect to both treatment conditions (table 9).
Figure 14. Quantitation of PCNA labeling in Jensen sarcoma. Percentage of cells labeled with an absence of the nucleolar region, percentage of cells exhibiting granular staining including the nucleolus, and percentage of cells exhibiting homogenous labeling. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the weighted mean ± SE of 3-8 separate fields comprising 100-250 cells.
Cells exhibiting homogenous labeling

Granular labeling
No nucleolar labeling

% Cells exhibiting

Time (Hr) 24 48 72 96 120

CONTROL  
CHARA  
DOX  

* * * * * *
Table 7. Pairwise comparison of the influence of time in Jensen sarcoma within control, Chara extract and doxorubicin treated cells for number of nucleolus-negative, granular and homogenous labeled cells.

<table>
<thead>
<tr>
<th>Group (Hrs)</th>
<th>Time</th>
<th>Nucleolus Negative</th>
<th>Granular</th>
<th>Homogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48  72  96  120</td>
<td>48  72  96  120</td>
<td>48  72  96  120</td>
</tr>
<tr>
<td>Con</td>
<td>24</td>
<td>S    NS  NS  NS</td>
<td>S    S    S    NS</td>
<td>S    S    S    NS</td>
</tr>
<tr>
<td>Con</td>
<td>48</td>
<td>-    S    S    S</td>
<td>-    NS  NS  S</td>
<td>-    S    S    S</td>
</tr>
<tr>
<td>Con</td>
<td>72</td>
<td>-    -    NS  NS</td>
<td>-    -    NS  S</td>
<td>-    -    NS  S</td>
</tr>
<tr>
<td>Con</td>
<td>96</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
</tr>
<tr>
<td>Chara</td>
<td>24</td>
<td>NS   S    S    S</td>
<td>NS   NS  NS  NS</td>
<td>NS   NS  S    NS</td>
</tr>
<tr>
<td>Chara</td>
<td>48</td>
<td>-    S    S    S</td>
<td>-    S    NS  NS</td>
<td>-    NS  S    S</td>
</tr>
<tr>
<td>Chara</td>
<td>72</td>
<td>-    -    S    S</td>
<td>-    -    NS  NS</td>
<td>-    -    S    S</td>
</tr>
<tr>
<td>Chara</td>
<td>96</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
</tr>
<tr>
<td>Doxo</td>
<td>24</td>
<td>S    NS  NS  NS</td>
<td>NS   NS  NS  NS</td>
<td>S    NS  NS  S</td>
</tr>
<tr>
<td>Doxo</td>
<td>48</td>
<td>-    S    S    NS</td>
<td>-    NS  NS  NS</td>
<td>-    S    S    S</td>
</tr>
<tr>
<td>Doxo</td>
<td>72</td>
<td>-    -    NS  NS</td>
<td>-    -    NS  NS</td>
<td>-    -    NS  S</td>
</tr>
<tr>
<td>Doxo</td>
<td>96</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
<td>-    -    -    -</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired points are indicated by (S), while non-significant comparisons are indicated by (NS).
Figure 15. Quantitation of PCNA labeling in Intestine 407. Percentage of cells labeling with an absence of the nucleolar region, percentage of cells exhibiting granular labeling including the nucleolus, and percentage of cells exhibiting homogenous labeling. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the weighted mean ± SE of 3-8 separate fields comprising 100-250 cells.
Table 8. Pairwise comparison of the influence of time in Intestine 407 within control, Chara extract and doxorubicin treated cells for number of nucleolus-negative, granular and homogenous labeled cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (Hrs)</th>
<th>Nucleolus Negative</th>
<th>Granular</th>
<th>Homogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48</td>
<td>72</td>
<td>96</td>
<td>120</td>
</tr>
<tr>
<td>Con</td>
<td>24</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Con</td>
<td>48</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Con</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Con</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chara</td>
<td>24</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Chara</td>
<td>48</td>
<td>-</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>Chara</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Chara</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Doxo</td>
<td>24</td>
<td>NS</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>Doxo</td>
<td>48</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Doxo</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Doxo</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P<0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
Figure 16. Quantitation of PCNA labeling in Chang liver. Percentage of cells labeling with an absence of the nucleolor region, percentage of cells exhibiting granular labeling including the nucleolus, and percentage of cells exhibiting homogenous labeling. Bars with an (*) indicate significant differences between treatment groups within each time frame as assessed by Duncan's multiple range test (P<0.05). Data represents the weighted mean ± SE of 3-8 separate fields comprising 100-250 cells.
% Cells exhibiting no nucleolar labeling

% Cells exhibiting granular labeling

% Cells exhibiting homogenous labeling

Time (Hr) 24 48 72 96 120

CONTROL

CHARA
Table 9  Pairwise comparison of the influence of time in Chang liver within control and Chara extract treated cells for number of nucleolus-negative, granular and homogenous labeled cells.

<table>
<thead>
<tr>
<th>Time Group (Hrs)</th>
<th>Nucleolus Negative</th>
<th>Granular</th>
<th>Homogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48  72  96  120</td>
<td>48  72  96  120</td>
<td>48  72  96  120</td>
</tr>
<tr>
<td>Con 24</td>
<td>NS  NS  NS  NS</td>
<td>NS  NS  NS  NS</td>
<td>NS  S  NS  S</td>
</tr>
<tr>
<td>Con 48</td>
<td>-  NS  NS  NS</td>
<td>-  NS  NS  NS</td>
<td>-  NS  NS  S</td>
</tr>
<tr>
<td>Con 72</td>
<td>-  -  NS  NS</td>
<td>-  -  NS  NS</td>
<td>-  -  NS  NS</td>
</tr>
<tr>
<td>Con 96</td>
<td>-  -  -  S</td>
<td>-  -  -  NS</td>
<td>-  -  -  NS</td>
</tr>
<tr>
<td>Chara 24</td>
<td>NS  NS  S  NS</td>
<td>NS  S  S  NS</td>
<td>S  S  S  S</td>
</tr>
<tr>
<td>Chara 48</td>
<td>-  NS  S  NS</td>
<td>-  NS  NS  NS</td>
<td>-  NS  S  NS</td>
</tr>
<tr>
<td>Chara 72</td>
<td>-  -  S  NS</td>
<td>-  -  NS  NS</td>
<td>-  -  S  NS</td>
</tr>
<tr>
<td>Chara 96</td>
<td>-  -  -  NS</td>
<td>-  -  -  NS</td>
<td>-  -  -  NS</td>
</tr>
</tbody>
</table>

Calculated using Duncan's multiple range test. Significant differences (P, 0.05) between paired time points are indicated by (S), while non-significant comparisons are indicated by (NS).
The application of Chara extract seemed to affect the overall pattern of PCNA labeling most strongly in Jensen sarcoma (figure 14 and table 7). This was particularly pronounced with respect to granular (active S-phase) labeling where the extract had a significant inhibitory effect relative to both control and doxorubicin treated cultures. Somewhat surprisingly, doxorubicin seemed to have a mild stimulating effect in Jensen sarcoma with regard to granular labeling, although this pattern was not uniform over time.

Despite some apparently significant differences with respect treatment or time factors no consistent trends were evident for controls, Chara extract or doxorubicin treatment in Intestine 407 or Chang liver. Indeed, Chara extract had virtually no effect on PCNA labeling in Chang liver and very little in Intestine 407 compared to controls.
DISCUSSION

The primary focus of this investigation was to further examine the effects of an extract from the alga Chara globularis on the growth of cancerous and non-cancerous cells in culture. The approach taken was to compare the effects of such extracts relative to those of the well characterized anti-neoplastic agent, doxorubicin. Several cell cycle and transcription-related indicators were monitored for this purpose, including cell morphology, growth kinetics, the appearance of coiled bodies, nucleoli and PCNA labeling. These parameters were examined in relation to cell type, time in culture and treatment with anti-neoplastic agents.

Cell Proliferation and Morphology

The results of the present investigation clearly demonstrated that addition of 5% v/v Chara extract markedly inhibited the growth of Jensen sarcoma cells in culture. Indeed, the extract appeared to be both more effective and selective in this regard than 5nM doxorubicin. For example, not only was inhibition of Jensen sarcoma by Chara extract faster than doxorubicin (24 vs 48 hrs after treatment), it was also less pronounced than doxorubicin in the non-cancerous, intestine 407 cells. Collectively, therefore, these results strongly support previous reports (Gallo, personal communication; Sherif, 1980) that the alga Chara contains compounds with anti-neoplastic properties.

Application of Chara extract did significantly affect cell density at the plateau phase in the two non-cancerous cell lines, though far less dramatically than Jensen sarcoma. This may well reflect cytotoxic activity resulting from accumulation of extract-derived, secondary metabolites that
only reached critical concentrations as the cultures approached confluence (Dolfini et al., 1973).

While Chara extracts appeared to preferentially inhibit growth of Jensen sarcoma, doxorubicin inhibited the growth of both Jensen sarcoma and Intestine 407. This observed action by doxorubicin on both cancerous and non-cancerous cells has also been reported in other studies (Tidefelt et al., 1991). Although the precise reason for this is unclear, doxorubicin may have a broad spectrum inhibitory action on cell growth compared to Chara extract.

Unlike the non-cancerous cells which did not exhibit any gross alterations in morphology following prolonged treatment with either Chara extract or doxorubicin, Jensen sarcoma cells exhibited hyperplasia along with an increase in cytoplasmic extensions. Such gross changes in morphology in cancerous cells may be a prelude to eventual resistance against anti-neoplastic agents, through induced differentiation at the nuclear, cytoplasmic, or membrane levels.

There are numerous reports in the literature that cancer cells resistant to anti-neoplastic agents exhibit one or more of the following characteristics: a) differentiated morphology due to changes in expression of adhesion and membrane proteins, b) increased expression of P-glycoprotein which is involved in the efflux of drugs from the intracellular region, c) slower rates of growth, and d) increased expression of enzymes involved in the metabolism of anti-neoplastic agents such as glutathione-S-transferase (Gibelli et al., 1994; Rasbridge et al., 1994; Choa et al., 1992). Agents have also been found that can redirect cellular differentiation by binding to actin and inducing polymerization or stabilization of pre-existing actin filaments (Bubb et al., 1994). Amine oxides reportedly induce changes in molecular organization, osmotic and
permeability characteristics of cell membranes (Miko and Devinsky 1992), and retinoids have been found to modulate gene expression by binding to RAR chromosome sites (Pemrick et al., 1994).

Fibrillarin Containing Structures

Despite significant changes in CB frequency and PCNA staining patterns (see discussion below), nucleolar components as revealed by immunofluorescence labeling with anti-fibrillarin antibody 72B9, remained comparatively stable. This overall uniformity was evident among all three cell lines, both in terms of treatment regime and age in cultures. Since fibrillarin is a major structural protein of pre-nucleolar bodies and nucleoli (Oches et al., 1985; Spector, 1993) these observations indicate that the structural integrity of these key nuclear domains was relatively unaffected by the diverse growth and treatment conditions utilized in this investigation. However, it remains unclear whether such stability also expanded to the functional aspects of these domains, namely rRNA metabolism and pre-ribosome production.

Any numerical and morphological assessment of fibrillarin containing nuclear structures based on light microscopy is clearly limited in resolution and should be extended to the electron microscope level. Moreover, inclusion of all fibrillarin positive elements in this analysis (pre-nucleolar bodies, nucleoli, CBs and other smaller components), could well mask time and/or treatment effects on each individual structure. That was certainly the case with respect to fibrillarin negative CBs (see discussion below), but it is also known that the size and shape of nucleoli vary with respect to the cell-cycle. During G1, nucleoli are characterized as small segregate structures possibly
coalescing into larger more prominent nucleoli during G2 (Spector, 1993). Such effects may not have been as pronounced in the present study which utilized asynchronous cells.

In the present study, the apparent absence of major structural effects on fibrillarin containing nuclear structures in terms of treatment could indicate that rRNA transcription was insensitive to the concentrations of Chara extract or doxorubicin employed. For example, immunofluorescent labeling of cells treated with the transcriptional inhibitors, actinomycin D or 5,6-dichloro-1-beta-d-ribofuranosyl benzimidazole which selectively block rRNA production, induced segregation of nucleoli into remnant structures (Raska et al, 1990). That was clearly not observed here. Along related lines, while RNA polymerase II and III have been shown to be sensitive to doxorubicin treatment (Chaung and Chuang, 1979 and Logan and Ackerman, 1988), RNA polymerase I, which is responsible for rRNA transcription, appears to be insensitive to this agent at micromolar concentrations (Wassermann et al, 1988). Nonetheless, doxorubicin can induce nucleolar segregation at relatively high micromolar concentrations (Abe et al, 1996), indicating that its precise mode of action in this regard remains to be determined.

Coiled Bodies and Cell-Cycle

The results of the present investigation are in general accord with many previous studies on cells in cultures. (For Review: Brasch and Ochs, 1992; Lamond and Carmo-Fonseca, 1993), showing that CB numbers can vary in relation to growth and metabolic rates and stages of the cell-cycle. Beyond that, however, significant differences were observed among the three cell lines utilized here. Not only were the
absolute numbers of CBs different, lowest in Jensen sarcoma and highest in Chang liver, but their relative frequency over time also varied significantly. For example, in Jensen sarcoma control cultures, only an average of two cells in ten displayed CBs at 24 hrs, while at 72-96 hrs virtually all cells did. In sharp contrast, Chang liver averaged 2.4-3.2 CBs for the duration and intestine 407 values were intermediate. Since the accompanying western blot data did not reveal comparable quantitative differences in total cell p80-coilin levels, the observed differences in both absolute and relative CB numbers must be due to, as yet, unknown causes.

An unexpected and potentially very interesting correlation emerged when CB number changes and PCNA labeling patterns were compared. For all three control cultures, the pattern of CB development over time, appeared to parallel most closely the development of nuclei exhibiting granular labeling which corresponds to those active in DNA synthesis (Bravo and Macdonal-Bravo, 1985). This does not imply that more CBs developed during S-phase compared to either G1 or G2, since this study utilized asynchronous cultures. Actually the opposite is observed in synchronous cultures where cells in S-phase exhibit the fewest CBs compared to G1 or G2 (Andrade et al, 1991).

The addition of Chara extract to the non-cancerous cells did appear to depress CB development slightly, particularly in Chang liver during periods of logarithmic growth and near plateau, while not altering the relative proportion of various PCNA labeling patterns compared to controls. This suggests that Chara extract did slightly modify the transcriptional state of the non-cancerous cells, independent of changes in proliferation. The reason for these decreases is unknown, but could reflect interference in a signal transduction pathway regulating transcription or the
transcriptional process itself. For example, extracts from wheat germ have been shown to interfere with RNA polymerase III activity in yeast (Furter and Hall, 1991), while extracts from Koelreutera henryi and Polygonum cuspidatum have been observed to alter protein kinase activity (Chang et al., 1996). Alternatively, the observed depression in CB development may not be due to the extract itself, but a possible accumulation of secondary metabolites as mentioned previously which may be affecting the cell density attained by the non-cancerous cells.

While Chara extract appeared to exhibit little effect on the cell-cycle of the non-cancerous cells, it had a dramatic effect in Jensen sarcoma. For example, the significant increase of Jensen sarcoma cells exhibiting no nucleolar labeling along with the significant decreases of cells with granular or homogenous labeling, suggests that the extract was blocking cells at the late G1 or early S-phase. This accumulation of cells exhibiting one PCNA labeling pattern has been seen with drugs such as hydroxyurea that block S-phase initiation and this results in an increased number of cells exhibiting no nucleolar labeling (Bravo and MacDonald-Bravo, 1985). Thus, the decreases seen in CB numbers in Chara treated Jensen sarcoma compared to controls, may be a reflection of a decreased rate of proliferation, as assessed by a decrease in the proportion of cells exhibiting granular labeling. However, other effects of Chara extract on transcription, such as those previously mentioned for the non-cancerous cells, may also be involved.

The addition of doxorubicin resulted in dramatic decreases in CB development in Jensen sarcoma and modest changes in Intestine 407, while altering the appearance of granular labeled Jensen sarcoma and slightly altering them in intestine 407. These decreases in CB development suggest that doxorubicin may be interfering with the transcriptional
process by intercalating within the major groove of DNA, thus, inhibiting RNA polymerase (Logan et al., 1989). However, while doxorubicin depressed CB development in Jensen sarcoma, it appeared to increase the proportion of cells exhibiting a granular labeling pattern compared to control Jensen sarcoma. Therefore, the intercalation of doxorubicin within the DNA may also result in a delay in the progression of DNA synthesis, for example, by inhibiting the helicase progression (Bachur et al., 1992). However the absence of a decrease of homogenously labeled cells representing G1 and G2 phase, suggests that this might not be the case. Instead these increases in granular labeled Jensen sarcoma may be a reflection of increased association of PCNA with DNA repair sites due to DNA damage induced by doxorubicin (Celis and Madison, 1986; Toschi and Bravo, 1988). In contrast to Jensen sarcoma, doxorubicin treated Intestine 407 exhibited an initial decrease in the proportion of cells in active S-phase. In conjunction with the decrease and shift in CB development over time, doxorubicin may also have exerted an initial cytotoxic effect on intestine 407.

A surprising observation was that Jensen sarcoma cells exhibited a high proportion of no nucleolar PCNA labeling, while Intestine 407 exhibited the highest proportion of homogenous labeling and Chang liver appeared to be fairly evenly mixed between the homogenous and no nucleolar labeling. The significance for these differences is unknown, but they may reflect changes in the stages of transformation from normal to cancerous cells. The cancerous Jensen sarcoma cells may be primed for entering DNA synthesis phase by having cells at the late G1 and early S-phase, while the non-cancerous cells are set to maintain their status in either G2 or, more likely, G1. These differences in the proportion of cells exhibiting various PCNA labeling patterns could be
explained by variations in the duration of each part of the cell-cycle (Takahashi et al., 1995).

A major and somewhat unexpected finding in the present study was the presence of two distinct populations of CBs, one containing and one apparently lacking fibrillarin. This was revealed by double-labeling experiments using antibodies against both p80-coilin and fibrillarin. While it is possible that these "fibrillarin-negative" CBs are truly devoid of this protein, it is more likely that the appropriate fibrillarin epitope was inaccessible to the anti-fibrillarin antibody 72B9. Either way, this might indicate a structural or conformational difference reflective of two distinct CB sub-populations. Whether this also indicates possible functional differences between the two types of CBs, and thereby account for their observed differences in behavior in all cell cultures with respect to time and treatment regime, is as yet unclear.

The association of fibrillarin with CBs has been reported before and has suggested a possible functional relationship between nucleoli and CBs (Raska et al., 1990; Raska et al., 1991; Ochs et al., 1994). The significance of these two possible classes of CBs is unknown, however they may represent respective changes in nucleolar and extranucleolar gene expression (Malatesta et al., 1994).

The addition of either doxorubicin or Chara extract had a generally negative effect on both classes of CBs in Jensen sarcoma. In Intestine 407, addition of Chara extract appeared to affect CBs more negatively lacking fibrillarin, while doxorubicin had a mixed effect. In Chang liver, addition of Chara extract appeared to have a more negative effect on fibrillarin positive cells compared to those without detectable fibrillarin, particularly as the cells approach confluency.
A unique observation about the proportion of "fibrillarin-positive" and "fibrillarin-negative" CBs was made (figure 8, 9 and 10). For the most part, in all of the cell lines, the proportion of these two CB subpopulations appears to be insensitive to drug exposure with either Chara or doxorubicin when using the concentrations employed. This suggests that the relative proportion of these two classes of CBs within the mammalian nucleus may be tightly regulated.

The functional significance of the two CB subpopulations and their apparent differences relative to treatment with Chara extract and doxorubicin remains unclear. Carmo-Fonseca et al. (1992) have shown a decreased association of snRNP's in CBs, while the association of fibrillarin with CBs increases, in Hela cells treated with α-amanitin or heat shock. The change observed here may be similar in nature.

Additional Modes of Action for Chara extract Components

In Jensen sarcoma, Chara extracts appeared to be altering CB numbers by influencing cell-cycle behaviors, compared to doxorubicin which appeared to be directly potent on CB development by inhibiting transcription. The data from this study suggest that Chara extract exert its action by blocking or delaying the cells progression through late G1 or early S-phase as assessed by PCNA labeling (Bhuyan et al., 1972). This observed action may be exerted by changes related to the membrane or cytoskeleton. In the alga Chara, the internal space of antheridial cells become filled with polysaccharides (Gosek and Kwiatkowska, 1993; Gosek and Kwiatkowska, 1991). These polysaccharides could bind to specific membrane proteins which are expressed in neoplastic cells but expressed at a low level or not at all in non-cancerous cells. The result would be to increase the adhesiveness of the cells or block activation of membrane
bound receptors (Boyd and Read, 1988). In recent years, algal polysaccharides have been shown to represent an important class of products which exhibit various antitumor, anticoagulant, antithrombotic and antiviral activity (For review: Wong et al., 1994; Lincoln et al., 1991). The action of Chara extract may be similar to that seen by sulfated polysaccharides from Ascophyllum nodosum, which block non-small-cell bronchopulmonary carcinoma in the G1 phase (Riou et al, 1996). Similarly, Chara contains profilins which are actin binding proteins that help regulate the polymerization of actin. These profilins have been shown to exhibit a high homology with plant profilins which have been shown to bind to actin in cardiac muscle (Ruhlandt et al., 1994), thus a potentially interfering with cytoskeletal arrangements. Either one of these mechanisms could cause a decreased rate of proliferation and, thus, a corresponding decrease in rate of transcription resulting in a decrease in CB development. This is, however, assuming that only a single agent is responsible for the observed action of Chara on the growth of Jensen sarcoma in vitro and in vivo. Possible additive or synergistic effects between various agents could play a role as well. Either way, the data do appear to show that Chara naturally contains agents which exhibit fairly selective growth inhibitory properties.

Conclusion

This study does show that Chara globularis contains compounds which exhibit fairly selective anti-neoplastic activity. The approach to assess Chara's potential anti-neoplastic activity is unique, however, the approach does look at two factors which are usually altered in cancer cells, transcription and cell-cycle. This approach also provided additional insight into the nature of nuclear
organisation and how it is influenced by age in culture and growth inhibitors. Additional experiments are required to fractionate the extract in order to illucidate the actual agent(s) involved. However, it is speculated that the active agent in *Chara globularis* is a type of polysaccharide. Such anti-cancer agents may provide new approaches in treating cancer, since conventional means usually carry undesirable side effects. Polysaccharides are water soluble and easily administered. In addition they have not been found to induce mutagenic or carcinogenic effects (Wagner, 1985). Bioactive polysaccharides from algae have also been shown to induce the production of various anti-tumor cytokines with less severe side effects associated with direct administration of recombinant cytokines (Lenk et al. 1989). Besides cytokine production, these polysaccharides have been shown to stimulate various immune cells as well. Future knowledge of the structural conformation of such compounds may also help lead to the development of more potent and specific compounds in the treatment of cancer.
REFERENCES


Celis, J.E., Bravo, R., Mose, L.P., and Fey, S.J. 1984. Cyclin: A nuclear protein whose level correlates directly with the proliferative state of normal as well as transformed cells. Leukemia Res. 8:35-44.


Gallo, D. Personal Communication, California State University, San Bernardino.


84


