UNIVERSITY OF JYVÄSKYLÄ

Morphological characterization of neuronal and non-neuronal phenotypes of the human neuroblastoma SH-SY5Y cell line

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Master's Thesis

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Cell Biology

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Preface

This study was done at the University of Jyväskylä at the Department of Biological and Environmental Sciences. The experiments were done in 2007 from late spring to early summer, and the writing and analysis of the results were done in spring 2008.

I thank my supervisor docent, Ph.D., Tuula Jalonen for the faith in my ability to do this study and for the friendship that developed between us during my studies at the University of Jyväskylä. I also thank my other supervisor M.Sc. Riina Sarkanen for advice during my experiments and for helping me during writing. The laboratory technicians Pirjo Kauppinen, Arja Mansikkaviita and Eila Korhonen have been extremely helpful and patient with me and my never-ending questions. My fellow student Reija Silvennoinen also deserves special thanks for the priceless help during the experiments, for listening my moaning and coping with my bossing without any complain. I also give my big thanks to M.Sc. Anna Mäkelä for the media, Ph.D. Hilkka Reunanen for the PAP pen, special laboratory technician Paavo Niutanen for the help with the microscopes and M.Sc. Kirsi Pakkanen for the help in finding all needed during the experiments.

I also give special hugs for all my friends for the support during the stressful and not-so-stressful times in my life, especially to the “Pimp My Lab team” for the encouraging words. My Mom Mailis Salo-Roiha and Dad Timo Roiha have always been my support, and have a never-ending faith in me, and I want to thank them with love. I also thank my brothers Pasi Viitasaari and Hannu Viitasaari for the support in the form of brotherly words and hugs. And finally, I want to thank my fiancé Tarmo Koponen for the patience and help with my no-know-how with computers and programs, for loving and supporting me, even when I was cranky with him because of the problems with the experiments.

Tiivistelmä

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Tiivistelmä:
Ihmisen neuroblastooma on pahanlaatuinen ja huonoennusteinen lapsilla esiintyvä syöpä. Se koostuu kolmesta eri solutyypistä: hermosolun kaltainen N-tyypin solu, epiteelisolun kaltainen S-tyypin solu ja välimuotoinen I-tyypin solu. Näillä solutyypeillä kullakin on sille ominaiset molekylaariset ja morfologiset piirteet, ja eri solujen esiintymisen kasvaimessa on esitetty liittyvän neuroblastooma syövän pahanlaatuisuuteen. Tässä tutkimuksessa ihmisen neuroblastooma SH-SY5Y -solulinjasta erotettiin N- ja S-tyypin solut ja tätä varten kehitettiin uusi metodi. SH-SY5Y-soluja sekä kantalinjasta erotettuja N- ja S-tyypin soluja käsiteltiin retinolihapolla, kolesterolilla ja mangaanilla eri vasteiden löytämiseksi. Soluja tarkasteltiin valo- ja fluoresenssimikroskopian avulla ja tulokset analysointiin tilastollisesti. Retinolihappo on A-vitamiinin johdannainen, jota käytetään solujen erilaistajana myös syöpähoidoissa. Koska retinolihapolla on vähäinen apoptoottinen vaikutus soluihin ja kolesterolin on esitetty olevan hyödyksi solujen erilaistumisessa, käsiteltiin soluja retinolihapolla ja kolesterolilla sekä niiden yhdistelmällä. Tämän lisäksi mangaanin aiheuttamaa solukuolemaa tutkittiin retinolihapon ja kolesterolin läsnäollessa. Kehitetty uusi N- ja S-tyypin solujen erottelumenetelmä osoittautui tehokkaaksi ja nopeaksi. Verrattaessa eri solutyyppejä toisiinsa huomattiin, että retinolihapolla ei ollut vaikutusta S-tyypin soluihin. Tämä saattaa johtua siitä, että ne lienevät neuroblastooman erilaistuneimpia soluja. S-tyypin solut vastustivat parhaiten mangaanin aiheuttamaa solukuolemaa. Tämä saattaa vahvistaa oletuksen, että juuri nämä solut ovat vastuussa syövän uusiutumisesta. Kolesteroli auttoi soluja erilaistumaan ja suojeli soluja retinolihapon ja mangaanin apoptoottiselta vaikutukselta. Retinolihappo sen sijaan teki solut alttiimmaksi mangaanin aiheuttamalle apoptoosille. Neuroblastooma-solulinjan lisäksi tutkimuksessa käytettiin ihmisen astrosytooma U373-MG -solulinjaa verrokkina kaikissa käsittelyissä.

Avainsanat: apoptoosi, astrosytooma, erilaistaminen, GFAP, kolesteroli, mangaani, morfologia, neuroblastooma, retinolihappo, vimentiini

Abstract

University of Jyväskylä **Abstract of Master´s Thesis**
Faculty of Mathematics and Science

Author:	Taina Roiha
Title of thesis:	Morphological characterization of neuronal and non-neuronal phenotypes of the human neuroblastoma SH-SY5Y cell line
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Abstract:
Human neuroblastoma is a malignant childhood cancer with poor prognosis. The tumor is heterogeneous, consisting of three cell types (neurilemmal N-type, epithelial-like S-type and intermediate I-type), which have been observed both *in vitro* and *in vivo*. These phenotypes have distinct molecular properties and they have been suggested to contribute to the malignancy of the tumor. In this study an advanced method for separating the N- and S-type cells from parental SH-SY5Y human neuroblastoma cell line was introduced, and their responses to various treatments were observed using light and fluorescence microscopy, and statistical analysis. Retinoic acid is a derivative of vitamin A and its ability to cause differentiation has been used in cancer therapies. As retinoic acid has a minor apoptotic effect and cholesterol has been proposed to support cell survival and differentiation, treatments of the parental cell line and newly separated N- and S-type cells with retinoic acid, cholesterol, and retinoic acid together with cholesterol, were introduced. The survival of these cells was also studied with manganese treatments combined with the retinoic acid and cholesterol treatments. Manganese was chosen because it induces apoptosis and its distinct effect on different cell types could be an important factor in cancer treatments. The new advanced separation method provides an efficient way to study the distinct behavior of the N- and S-type cells *in vitro*. Retinoic acid was found to have no effect on the S-type cell morphology, which suggests that these cells are already differentiated. Also, the S-type cells were most resistant to the manganese-induced cell death, which suggests that these cells might be behind the poor prognosis of human neuroblastoma and the often observed reoccurrence of the tumor. Cholesterol protected the cells from retinoic acid and manganese-induced apoptosis. The U373-MG human astrocytoma cell line was used as a reference CNS tumor cell line.

Keywords: apoptosis, astrocytoma, neuroblastoma, cholesterol, differentiation, GFAP, manganese, morphology, retinoic acid, vimentin

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Abbreviations

ABC-transporter	ATP-Binding Cassette transporter
ApoE	Apolipoprotein E
ATCC	American Type Collection of cell Cultures
BSA	Bovine Serum Albumin
Chol	Cholesterol
CNS	Central Nervous System
CRABP	Cellular Retinoic Acid Binding Protein
DIV	Days *In Vitro*
DMT1	Divalent Metal Transporter 1
ECACC	European Collection of Cell Cultures
EDTA	Ethylene Diamine Tetraacetic Acid
ER	Endoplasmic Reticulum
F-12K	Kaighn's modification solution
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
LDL	Low Density Lipoprotein
MEM	Modified Eagle Medium
MnSOD	Manganese Super-Oxide Dismutase
NEAA	Non-Essential Amino Acids
NF68	Neuro-Filament protein 68
PBS	Phosphate Buffered Saline
PFA	ParaFormAldehyde
RA	Retinoic Acid

RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Responsive Element
RT	Room Temperature
RXR	Retinoid X Receptor
SE	Standard Error
WHO	World Health Organization

1. Introduction

Human neuroblastoma is one of the most malignant cancers occurring in children. It is not yet clear how and when it develops during embryogenesis, but it is thought to be a stem cell cancer occurring during autonomic nervous system development (for a review see McConville and Forsyth, 2003). Heterogeneous cell population is typical for neuroblastoma and it is seen both *in vivo* and *in vitro*. It has been suggested, that different cell types in human neuroblastoma express different degrees of malignancy, and have differential effect on the clinical outcome (Fidler, 1978; for a review see Castleberry, 1997). Human neuroblastoma cells have been used as a model for tumor cell differentiation and neuronal development, as well as a model for studies on neurodegenerative diseases (for a review see Abemayor and Sidell, 1989; Higashi et al., 2004).

Human astrocytoma (also known as glioblastoma or astroglioma) is a malignant cancer occurring at all ages, with poor prognosis (for a review see Preusser et al., 2006; Haque et al., 2007b). The origin of human astrocytoma is still unknown, but stem cells or progenitor cells have been proposed to be the source for this cancer (for reviews see Read et al., 2006; Quigley et al., 2007). Astrocytoma also manifests heterogeneity of cell types, though these different cell types are not as well studied as are the cell types of neuroblastoma (for a review see Miller and Perry, 2007). The reasons behind human astrocytoma are unknown, and though much used in studies, its characteristics are poorly known.

1.1. Human neuroblastoma and its cell phenotypes

One of the first studies describing human neuroblastoma was by Beckwith and Perrin (1963), and since then this cancer type has been widely studied. Despite of the years of research, it is still not known how this devastating tumor arises and how to effectively treat it. There are speculations about environmental factors associated with the occurrence of neuroblastoma, though no study has yet confirmed this. Neuroblastoma usually develops from postganglionic neurons in the preaortic ganglia and adrenal gland, and the heterogeneous cells in neuroblastoma reflect the whole spectrum of differentiated and undifferentiated cells of developing sympathetic nervous system (for a review see McConville and Forsyth, 2003).

Despite this heterogeneity, neuroblastoma formation is tightly regulated and cell lineage-dependent, as neuroblastoma never develops from neural precursor cells of other than sympathoadrenal origin (for a review see Nakagawara and Ohira, 2004). Human neuroblastoma has been classified according to the extent of the tumor to stages I-IV, and the survival of patients is largely age-dependent, younger children having better prognosis (Breslow and McCann, 1971). Most of the therapies for neuroblastoma include dose-intensive chemotherapy and surgery (for a review see Castleberry, 1997). The variability in human neuroblastoma may also be the reason for varying results obtained from different studies. Also, the differences observed in tumorigenicity of the human neuroblastoma cells reflect their phenotype. The S-type (epithelial-like) cells are non-malignant, the I-type (intermediate) cells are most malignant, and the N-type (neuronal) cells lay between these two forming slowly growing malignant tumors (Walton et al., 2004). One unanswered question is, whether these different cell phenotypes also play a role in tumor regression.

The cells lines derived from neuroblastoma tumor biopsies have been difficult to establish, because the cells form tight clusters and neither chemical, nor mechanical separation produces single cells for cell cultures (Schlesinger et al., 1976). However, different cell types have been obtained from established cell lines and their characteristics have been studied, despite the difficult maintenance of some of these isolated cell clones (Biedler et al., 1973; Ciccarone et al, 1989). Most of the cell lines derived earlier from the tumors consist of two types of cells; one which is a small, aggregate-forming and has short processes (N-type cell), and the other which is a large, flat and epithelial-like cell (S-type cell). When the cells have been cultured for a longer time, epithelial-like cells disappear from the culture and the neural type remains.

The cell lines established from tumors express different characteristics, including cell surface markers, neurotransmitter synthesis enzymes and odd chromosomes (Biedler et al., 1973; West et al., 1977; Biedler et al., 1978; Ciccarone et al., 1989). There is a larger variation between different established S-type cell lines than in different N-type cell lines, and the limited growth of S-type cells suggests, that these cells are more differentiated than N-type cells. In contrast to S-type cells, all established N- and I-type cell cultures have been immortal

from the start (Ciccarone et al., 1989). Despite these differential proliferation patterns of cell types, the N-type cells have been suggested to be the most differentiated cell type in the human neuroblastoma both *in vivo* and *in vitro*.

The cell types seen *in vitro* have been characterized as neuronal, neurilemmal (Schwann cells) and melanocytic cell types (Ross and Biedler, 1985; DeClerck et al, 1987; Rettig et al., 1987; Tsokos et al., 1987; Mora et al., 2001). Neuronal cells have processes and neurosecretory granules, neurilemmal cells are flat and non-pigmented, and melanocytic cells are flat and pigmented (Tsokos et al., 1987). It has been suggested that the Schwann-type cells observed in the neuroblastomas might be due to the chemoattractive factors produced by differentiating neuroblastoma (N-type) cells, and thus these cells would not be neuroblastoma cells (Ambros et al., 1996). At present, the neuronal and epithelial-like (previously named Schwann-like) phenotypes are called N- and S-type cells according to their morphology. The N-type cells are neuroblastic, contain noradrenergic biosynthetic enzymes, attach better to each other than to substrate and form pseudoganglia resembling aggregates (Ciccarone et al., 1989; for a review see Ross et al., 2003). The S-type cells are epithelial-like and do not have neuronal properties. They adhere strongly to substrate, show contact inhibition, and have tyrosinase activity which is a melanocytic property (Ciccarone et al., 1989; for a review see Ross et al., 2003). The N- and S-type cells can also spontaneously transdifferentiate to each other (Figure 1.).

Besides the N- and S-phenotypes, I-type cells have been characterized from human neuroblastoma (Figure 1). These intermediate cell types have characteristics of both N- and S-type cells, and they have been suggested to be the stem cell from which the N- and S-type cells arise (Ciccarone et al., 1989; Rettig et al., 1987; Ross et al., 1995; for a review see Reya et al., 2001; Walton et al., 2004).

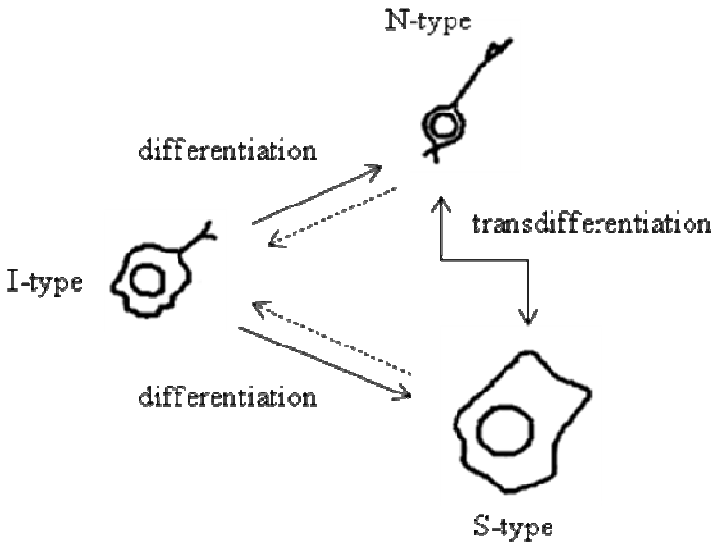

Figure 1. Proposed model for the different phenotype occurrence in the human neuroblastoma *in vivo* and *in vitro*. The I-type cell represents a stem cell, from which the N- and S-type cells arise. In cell cultures the N- and S-type cells can transdifferentiate to each other, and possibly back to I-type cell.

Biagiotti et al. (2006) divided the SH-SY5Y neuronal and epithelial-like cell phenotypes further into subgroups of N- and S-type cells (N_N-, N_S-, S_0-, S_1- and S_2-subtypes) according to time intervals when the morphological and electrophysical changes occurred. The S_0-subtype occurred soon after the isolation of different cell types and remained in the culture until day 60. After this the cells changed into S_1-subtype, and after 120-140 days S_2-subtypes appeared in the cell culture. The N_N-subtype was identical to the parental SH-SY5Y cell line and did not change its morphology. The N_S-subtype appeared in the S-type cell cultures, and these cells needed to be removed in order to allow the S-type cells to proliferate. Besides the morphological changes, the subtypes were distinct in their expression of ion channels, and the subtype conversions in the cultures were confirmed by these electrophysiological changes. In addition to characterization as N-, S- and I-type cells, an infrequently used way of classification, mainly used in diagnostics, is to divide the human neuroblastoma cells in undifferentiated, poorly differentiated and differentiated cell types (Shimada et al., 2001). Also, cells with different genetic markers and different amount of genetic changes from low to high have been classified into three different groups (Valent et al., 1999). These three groups may be the N-, S- and I-type cells presented above.

1.2. Human astrocytoma

Human astrocytoma is a malignant brain tumor, which is problematic in terms of therapy efficiency. The poor prognosis and insufficiency of chemotherapy makes this cancer challenging and important to study (Haque et al., 2007b). Human astrocytes are one of the few cells, which can proliferate in the adult human brain, but astrocytoma has been suggested to be formed from a neuronal progenitor cell, though the contribution of a specific stem cell has not been ruled out (for reviews see Preusser et al., 2006; Read et al., 2006; Quigley et al., 2007). Environmental factors and various genetic changes are also suggested to be involved in the formation of this cancer (for reviews see Stupp et al., 2007; Quigley et al., 2007). Astrocytoma can be found at any age, though the peak of this cancer is in adults over 40 years (for a review see Preusser et al., 2006).

Human astrocytomas are classified according to WHO (World Health Organization) to stages I-IV, where stage I represents low-grade glioma and stage IV most malignant glioma. The astrocytoma tumors can evolve over the years from low-grade glioma to a malignant tumor, or a malignant astrocytoma can be found directly. Unlike many other cancers, malignant human astrocytomas do not metastasize, though all patients with malignant astrocytoma will have reoccurrence of the tumor (for a review see Stupp et al., 2007). One of the reasons behind the poor prognosis is the high invasiveness of astrocytoma cells and their resistance to apoptosis (for reviews see Preusser et al., 2006; Furnari et al., 2007). As with human neuroblastoma, human astrocytoma prognosis is highly dependent on the age of the patient, older people being more susceptible (for a review see Stupp et al., 2007).

Human astrocytomas are also heterogeneous with three major types of cells; fibrillary, gemistocytic and giant cells (for a review see Miller and Perry, 2007). The fibrillary cells have large and irregularly shaped nuclei, scant cytoplasm and they form loose processes. The gemistocytic cells have rough nuclei, rich cytoplasm and they form short processes. The only occasionally found giant cells have a large and granular cytoplasm (for a review see Miller and Perry, 2007). These cell types vary especially in their amount of genetic changes, but these changes related to the different degrees of differentiation have not been studied in these human astrocytoma cell phenotypes (for a review see Quigley et al., 2007). Though many

neurological disorders include interactions with defective astrocytes, the neuron-glia interactions are mainly unidentified (for a review see Seifert et al., 2006).

1.3. Retinoids and retinoic acid in cell differentiation

Retinoids are natural and synthetic derivatives of vitamin A, and their differentiating effect is known (for a review see Abemayor and Sidell, 1989). Generally cancer cells are responsive to retinoic acid (RA), and this is the reason why retinoids are widely used in cancer therapies (for a review see Miller, 1998). The differentiating abilities of different isoforms of retinoids have been tested earlier (Ponzoni et al., 1995; Hewson et al., 2000). The most effective isoform is 9-*cis* retinoic acid, but it is toxic to cells (Hewson et al., 2000). All-*trans* retinoic acid has proven to be an effective differentiating agent, and only slightly toxic in relatively large dosages (Phuphanich et al., 1997). This makes all-*trans* retinoic acid a good candidate for inducing differentiation and to be specifically used in cancer therapies (Haque et al., 2007b). RA has been used with promising results, though the treatment does not provide a cure for cancer, and there are also unresponsive cases (Yung et al., 1996; Defer et al., 1997; Phuphanich et al., 1997). Treatment of human neuroblastoma cells with RA increases their survival from hypoxia (Hoehner and Prabhakaran, 2003). This suggests that RA treatment might, instead of disturbing cancer cells, enhance the cell survival, and thus the benefits of RA application *in vivo* in cancer therapies are still controversial. Furthermore, the retinoids are not effective alone, and additional agents need to be found for increasing the efficiency of the RA therapies for cancer (Preis et al., 1988; Defer et al., 1997).

Retinoic acid is lipofilic and it is transported into cells with the help of Cellular Retinoic Acid Binding Protein (CRABP), which is ubiquitously expressed in all cells (Haussler et al., 1983; for a review see Abemayor and Sidell, 1989). RA regulates target genes by binding to Retinoic Acid Receptors (RAR) and Retinoid X Receptors (RXR), which form heterodimers to become active in both human neuroblastomas and astrocytomas (Cheung et al., 1996; Hewson et al., 2000). This heterodimerized protein complex then binds to Retinoic Acid Responsive Element (RARE), which becomes transcriptionally active leading to cell differentiation (Joshi et al., 2006). Different subtypes of these receptors are expressed in different cell types, and this leads to distinct differentiation pattern. By transfecting cells with

these receptor subtypes, cells can effectively be induced to undergo neuritogenesis, or just to stop proliferating (Cheung et al., 1996). Especially human neuroblasts have been reported to be sensitive to retinoid treatments, though their responses are not uniform, and depend on the cell phenotype (for a review see Abemayor and Sidell, 1989; Tsokos et al., 1987; Voigt and Zintl, 2003; Joshi et al., 2006). The differences in responses might exist because of the difference in retinoid receptor expression (Cheung et al., 1996; Joshi et al., 2006; Joshi et al., 2007). The one known common response to retinoids is their effect to decrease cell proliferation in all neuroblastoma cell types, but very little is known about their effects on astrocytoma cells.

Generally RA induces the neuroblastoma cells to form longer neurites (which is a commonly used sign of neuronal differentiation), and change their morphology towards the small N-type cells (Preis et al., 1988; Ross et al., 1995). It has been shown by Barletta et al. (1997), that the SH-SY5Y human neuroblastoma cell line has reduced invasiveness when it is treated with RA for more than four days. However, if the SH-SY5Y cells are treated with RA for only a short time, especially the N-type cells enhance their migration and invasion without any effect on proliferation (Preis et al., 1988; Voigt and Zintl, 2003; Joshi et al., 2006). It has also been earlier shown that with large concentrations (10 µM) and long exposure times (over five days), most of the human neuroblastoma cell types convert into N-type cells, though a small number of S-type cells can be seen throughout the treatment (Ross et al., 1995; Walton et al., 2004; Sarkanen et al., 2007). However, morphology changes in the human neuroblastoma cells are seen with ten times, or even hundred times lower than 10 µM concentrations of RA (Haussler et al., 1983; Preis et al., 1988). Some of the S-type cells do not change their morphology, and if RA is removed from the culture medium, I-type cells can be seen (Ross et al., 1995). In the human astrocytoma cells the RA treatment changes the cell soma to become slim, induces formation of long processes and decrease cell proliferation, thus causing differentiation towards a reactive astrocyte-like cell (Haque et al., 2007a).

1.4. Cholesterol in central nervous system

Cholesterol (Chol) is an important major non-polar lipid in membranes and it acts as protein binding domain in the plasma membrane. Inside the cell, cholesterol is synthesized in the ER

(Endoplasmic Reticulum) and it seems to have a role in ER-Golgi trafficking. From there cholesterol is usually targeted to recycling endosomes, synaptic vesicles, mitochondria and plasma membrane (for a review see Ikonen, 2008).

In the Central Nervous System (CNS) lipoproteins (mainly Low Density Lipoproteins, LDL) are used to transport lipids and cholesterol between different cell types, though cholesterol is taken inside the cells also via ABC-transporters (ATP-Binding Cassette transporters, Albrecht et al., 2008). Once inside the CNS, cholesterol is not released because of the high turnover of cholesterol into myelin (for a review see Ikonen, 2008). However, little is known about the trafficking and compartmentalization of cholesterol in the cells of CNS as most of the previous studies on cholesterol have been made with fibroblasts. Earlier cholesterol studies with astrocytoma cells have only included experiments on oxidative stress (e.g. Girão et al., 2003; for a review see Ikonen, 2008). It is known, that cholesterol can be released from astrocytes in the CNS, and therefore astrocytes are beneficial for the maintenance of proper neuronal cholesterol concentration. Also, astrocytic supply of cholesterol has been shown to have an important role in the maturation of synapses (Barres and Smith, 2001; Göritz et al., 2005; Fünfschilling et al., 2007). Cholesterol has also been associated with neurodegenerative diseases, such as Alzheimer's disease, by functioning with ApoE (Apolipoprotein E), which has a role in regenerative processes in the CNS (for a review see Ikonen, 2006).

It has been reported, that during the clinical RA treatments cholesterol levels of the cancer patients are elevated, for as yet unknown reason (Yung et al., 1996). At present the only study available of the effects of cholesterol together with RA *in vitro* is by Sarkanen et al. (2007). In that study the authors describe the benefits of using cholesterol together with RA to support the survival of SH-SY5Y neuroblastoma cells when they are differentiated. Also, according to other studies, cholesterol has been shown to help the cells to avoid the apoptotic effect of RA (Ponzoni et al., 1995; Mäntymaa et al., 2000). The protective role of cholesterol could play a critical role in therapies against neurodegenerative diseases, as well as in applications in cancer therapies.

1.5. Manganese in central nervous system

Manganese (Mn) is an essential trace element, which as an overdose causes Parkinson's disease resembling manganism (Levy and Nassetta, 2003). The main routes by which manganese gets into the body and CNS include the digestive system and respiratory tracts. The transportation of manganese into cells is not well understood, but DMT1 (Divalent Metal Transporter 1) and transferrin, both of which carry many metals, are suggested to transport also manganese (Roth, 2006). In cells manganese functions as a cofactor for many enzymes (Welder, 1996). The reason behind the accumulation of manganese, especially into dopaminergic regions of brain, is unknown (Fitsanakis et al., 2006). It has been suggested, that manganese exerts its effects by causing oxidative stress in mitochondria, as they do not have outward transport mechanism for manganese, and by affecting the ER (Higashi et al., 2004; Dukhande et al., 2006). The molecular effects of manganese are studied but as yet poorly understood (for a review see Normandin and Hazell, 2002).

Based on earlier studies, glial cells are in the CNS the most resistant cells to the effects of manganese, and manganese causes morphological changes in astrocytes, resembling those observed in Alzheimer disease patients' astrocytes (Hazell et al., 2006). Manganese induces decline in cell viability in a dose-dependent manner in both the human neuroblastoma and astrocytoma cells (Yin et al., 2008). It has been reported, that the human neuroblastoma cells are more vulnerable to manganese treatments than the different glial cells, and that astrocytes can take up more manganese than other cells in the CNS (Shukakidze et al., 2002; Higashi et al., 2004; Malthankar et al., 2004; Dukhande et al., 2006; Gunter et al., 2006). The manganese induced cell death reported by Dukhande et al. (2006) is predominantly necrotic, possibly because of a high manganese concentration (1 mM) used. However, results by another group suggest that the human astrocytoma cells die by apoptosis also with high manganese concentrations (Puli et al., 2006). In the manganese studies, various Mn concentrations, ranging from physiological (0.01 mM) to highly toxic (4.0 mM) concentrations, with many different exposure times, have been used, thus making the data highly inconsistent (Malthankar et al., 2004).

2. Aim of the Study

The main aim of this study was to find an efficient method for separating the N- and S-type cells from the parental SH-SY5Y human neuroblastoma cell line, and from each other in a way, that they do not transdifferentiate in the used cell culture conditions.

The second aim was to study if the newly separated N- and S-phenotypes, and the parental SH-SY5Y cells, respond differently on differentiation treatments with RA. As cholesterol has been shown to support the differentiation of the SH-SY5Y cells, additional treatments with cholesterol were introduced. Also the possible distinct survival of the different cell types from the manganese-induced cell death was observed.

Same experimental treatments introduced to the human neuroblastoma SH-SY5Y cells were applied also to the human astrocytoma U373-MG cells to further clarify the possible differences between neuronal and glial CNS tumor cells.

3. Materials and Methods

Changes in the human neuroblastoma SH-SY5Y and astrocytoma U373-MG cell morphology were studied by light and fluorescence microscopy, and the differential effects of RA, cholesterol and manganese on the proliferation and survival of these cells were evaluated.

3.1. SH-SY5Y cell culture and imaging

The human neuroblastoma SH-SY5Y cell line (originally from American Type Culture Collection, ATCC, CRL-2266, Manassas, VA, USA) was a kind gift from the University of Tampere, Departments of Cell Biology and Environmental Toxicology. The SH-SY5Y cell line and the separated phenotypes were cultured in 1:1 MEM (Modified Eagle Medium, 21090) and F-12K (Kaighn's Modification, 21127) media (GIBCO Invitrogen, Carlsbad, CA, USA) in 5 % CO_2 and + 37 °C. Medium was supplemented with 1 mM L-glutamine (Immuno Diagnostics, Wobum, MA, USA), Non-Essential Amino Acids (NEAA, 1 %, GIBCO), Fetal Bovine Serum (FBS, 10 %, PAA Laboratories, Pasching, Austria) and with streptomycin and ampicillin (1 %, PAA Laboratories). The details for the culture medium are presented in Appendix 7.1.

The SH-SY5Y cells of passages 25-31 used in this study were subcultured once a week, and the culture medium was additionally changed once a week. Cells were washed with PBS (Phosphate Buffered Saline) and trypsinized (trypsin-EDTA, Ethylene Diamine Tetraacetic Acid, GIBCO) for three minutes, after which the cell suspension was neutralized with culture medium, and cells were collected by centrifugation (1500 rpm for 3 min, Labofuge 400, Heraeus Instruments, Hanau, Germany). Cell pellet was resuspensed with culture medium and cells were counted by using hemocytometer (Marienfeld Laboratory glassware, Lauda-Königshofen, Germany). Cells were seeded with a density of 0.2×10^6 cells / 2 ml medium on 3 cm diameter dishes (Nunc, Rochester, NY, USA and Falcon, BD Biochemicals, San Jose, CA, USA). For 25 cm^2 and 80 cm^2 flasks (Nunc and Falcon) cells were seeded with a density of 0.5×10^6 cells / 10 ml medium and 2×10^6cells / 15 ml medium, respectively.

For morphological images Artcam-300MI camera (Olympus, Tokyo, Japan) connected to CX40 microscope (Olympus) was used and the images were taken by using QuickPhoto version 2.2 software (Promicra, Praha, Czech Republic). Picture editing was done with Photoshop C2S software (Adobe Systems, San Jose, CA, USA). All images in this study were taken randomly and editing was kept minimal.

3.2. N- and S-phenotype separation

The initial separation of the N- and S-type cells from the parental SH-SY5Y cell line were done by using a mechanical separation method proposed by Biagiotti et al. (2006). However, as the method was problematic and not as efficient as expected, a new method was developed.

Initially there were two dishes of SH-SY5Y parental cells, for which the slightly different separation methods were introduced. The separation was started when the parental cell line was at passage 26, and the cells were cultured for 7 DIV (Days *In Vitro*). For the future N-type cells a short trypsinization (1.5 min at RT, Room Temperature) was used to detach only the loosely adherent N-type cells. After this the N-type cell cultures were subcultured similarly to the parental SH-SY5Y cells, but the trypsinization time was for two minutes. To keep the cultures clean from the S-type cells, all cells in the culture were never fully detached.

For the future S-type cell culture, a very short trypsinization (1 min at RT) was first introduced to detach the N-type cells, which were carefully removed, and trypsinization was continued at +37 °C for additional three minutes, to detach the tightly adherent S-type cells. After this the S-type cells were subcultured similarly to the parental SH-SY5Y cells, but with initial trypsinization for one minute to remove the N-type cells, followed by a longer final trypsinization for four minutes. Imaging was performed as presented in section 3.1.

3.3. U373-MG cell culture

The human astrocytoma U373-MG cell line (originally from European Collection of Cell Cultures, ECACC, No: 89081403, Wiltshire, UK) was a kind gift from the University of Tampere, Departments of Cell Biology and Environmental Toxicology and in this study cell

passages 187-196 were used. The cells were cultured in MEM (21050, GIBCO) supplemented with sodium (Na) pyruvate (1 %, GIBCO), 1 mM L-glutamine (Immuno Diagnostics), NEAA (1 %), FBS (10 %) and 1 % antibiotics (all from PAA laboratories) in 5 % CO_2 and + 37 °C. Details for the culture medium are presented in Appendix 7.2.

Cells were subcultured every fourth day with trypsinization for seven minutes. The number of cells seeded for different plates was half of that used for the SH-SY5Y cells: for the 3 cm diameter culture dishes 0.1×10^6 cells / 2 ml medium, for 25 cm^2 and 80 cm^2 flasks 0.25×10^6 cells / 10 ml medium and 1×10^6 cells / 15 ml medium, respectively. All dishes are from Nunc and Falcon and the cell images were taken as described in section 3.1.

3.4. Calculation of cell proliferation

A hemocytometer (Marienfeld Laboratory) was used for counting the SH-SY5Y and U373-MG cells. Cells were cultured in 3 cm diameter cell culture Petri dishes and trypsinized, centrifuged and resuspensed in 1 ml of culture medium as presented in 3.1. According to the manufacturer, the 3 cm diameter dishes have an area of 8.8 cm^2, and the following equations were therefore used:

$$\text{number of cells in a dish} = \text{cells/ml} \times 1\ \text{ml}$$

$$\text{cells/cm}^2 = \frac{\text{number of cells in a dish}}{8.8\text{cm}^2}$$

Cells were first counted after 24 hours and thereafter in time points of two, four, six, eight and ten days. For each counting a new Petri dish of cells was used. The statistical analysis was done by using OriginPro 7.5 software (OriginLab, Northampton, MA, USA) for the independent *t*-test (significance level $P < 0.05$) and linear regression.

3.5. Immunocytochemistry

The intermediate filaments of the astrocytoma U373-MG and different cell types of the SH-SY5Y neuroblastoma cells were labeled with vimentin (1:100, anti-vimentin from goat, V 4630), NF68 (1:1000, monoclonal anti-NeuroFilament 68 from mouse ascites fluid, N 5139)

and GFAP (1:200, anti-Glial Fibrillary Acidic Protein from rabbit antiserum, G 9269. The nuclei were stained with Hoechst 33258 (1:1000) to show the presence of apoptotic nuclei (all primary antibodies and nuclei stain are from Sigma Aldrich, St Louis, MO, USA).

Cells were grown in multiwell chamber slides (Lab-Tek™ Permanox Multiwell, Nunc) 9 000 cells / 0.5 ml medium for the SH-SY5Y cells and 4 500 cells / 0.5 ml medium for the U373-MG cells, and quickly washed twice with PBS before fixation with 4 % PFA (paraformaldehyde, Merck, Whitehouse Station, NJ, USA) for 30 minutes. After fixation, the cells were washed with PBS three times for five minutes each. Cells were permeabilized with 0.5 % Triton-X (Sigma Aldrich) in PBS for 15 minutes, after which the chamber frame was carefully removed. Cells were again washed with PBS three times for five minutes each. After this, the chambers were marked using a PAP pen (Abcam, Cambridge, UK) and unspecific binding sites were blocked with 10 % Bovine Serum Albumin (BSA, Sigma Aldrich) for 30 minutes (100 µl for each chamber). Blocking solution was carefully removed and primary antibodies were added (100 µl for each chamber) for three hours at RT or overnight at + 4 ºC. The primary antibody solutions were prepared in 1 % BSA.

After incubation the cells were washed with PBS three times for ten minutes each and the secondary antibodies were added (1:200, 100 µl for each chamber): Alexa Fluor 488 IgG goat anti-mouse (A-11029), Alexa Fluor 546 IgG rabbit anti-goat (A-21085) and Alexa Fluor 633 IgG goat anti-rabbit (A-21071). All secondary antibodies are from Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA. The solutions were prepared in 1 % BSA and cells were incubated in dark for 90 minutes at RT. After this, cells were further washed with PBS three times for five minutes each, and nuclei were labeled using Hoechst 33258 for five minutes at RT (100 µl for each chamber). Cells were again washed with PBS six times for five minutes each and embedded by using Mowiol-Dabco (Sigma Aldrich). Cells were incubated at least overnight at + 4 ºC before imaging and after that stored horizontally at the same temperature in dark.

Fluorescence images were taken by using Leiz DMRPE camera (Leica, Solms, Germany) connected to Axiovert 25 microscope (Zeiss, Jena, Germany) with Spot Advanced

software (Diagnostics Instruments, Sterling Heights, MI, USA). Picture editing was done with Photoshop C2S software (Adobe Systems) and editing was kept minimal.

3.6. Retinoic acid and cholesterol treatments

The all-*trans* retinoic acid (MP Biomedicals, Solon, OH, USA) and cholesterol (3β-hydroxy-5 cholestene, Merck) stock solutions were made by dissolving both RA and cholesterol in 96 % ethanol to give 5 mM and 10 mg/ml solutions, respectively. The final ethanol concentration of less than 0.1 % used in this study has no effect on cell viability (Sarkanen et al., 2007). For the RA treatments, a concentration of 5 µM was used, for cholesterol treatments a concentration of 10 µg/ml was used, and for RA + chol treatments a solution with 5 µM RA and 5 µg/ml cholesterol was used (Sarkanen et al., 2007). Cells were exposed to RA and cholesterol starting the next day after subculturing, and cells were cultured for 4, 7 and 10 DIV before fixing and imaging. All cultures were kept in dark during the treatments. Cell counting was done at time points of 4, 7 and 10 DIV as described in section 3.4 and fluorescence labeling was done as presented in section 3.5.

3.7. Manganese treatments

Mangan (II) chloride-4-hydrat ($MnCl_2 \cdot 4H_2O$, Merck) was used in these experiments. Before experiments, a stock solution of 50 mM was prepared in PBS, of which an apoptosis-inducing concentration of 500 µM was used for experiments (personal communication, Prof. M. Aschner, Vanderbilt University, Nashville, Tennessee, USA; Yin et al., 2008). Manganese was added when cells were at 6 and 9 DIV and cultures were then left with manganese for another 24 hours before imaging. For cell counting, the cells were cultured for 10 DIV to allow proper differentiation with RA and full effect of cholesterol. Fluorescence labeling was done as presented in section 3.5. Cell death was evaluated by the occurrence of plasma membrane blebbing, detachment of the cells from the culture dish, by rounded cells and apoptotic nuclei (Bunone et al., 1997; Bian et al., 2002).

4. Results

An advanced method for separating the N- and S-type cells from the parental SH-SY5Y cell line was developed in this study. With the new method the neuronal N-type and non-neuronal S-type cells were morphologically different already after two subcultures, and the responses on RA, cholesterol and manganese treatments were distinctly different. The S-type cells were unresponsive to the differentiating effect of RA and cholesterol made the cells more stable in RA treatments. Cholesterol also seemed to help the SH-SY5Y and U373-MG cells to survive from the manganese-induced apoptosis.

4.1. Initial experiments for phenotype separation

In the initial experiments for separating the different phenotypes from the parental human neuroblastoma SH-SY5Y cell line, a mechanical method introduced by Biagiotti et al. (2006) was first used. The separated N- and S-type cell cultures were observed regularly, and after the second subculture the differences between the cell types were not yet clear, though they seemed to be morphologically distinct from the parental SH-SY5Y cell culture. In the N-type culture the cells grew in aggregates, in the S-type cell culture the cells were scattered, and there were plenty of dead cells in both cultures.

In the third subculture differences between the N- and S-type cell cultures became clearer and both cells grew relatively evenly. The N-type cells were smaller in size and appeared to have more neurites compared to the S-type cells, which were flat and larger. After this subculture, the morphological differences became more obvious, though separation was still not complete. Furthermore, there were still a large number of dead cells in the N-type cell culture. After these initial experiments, a new and more efficient method was developed to obtain faster results for separating the N- and S-type cells.

4.2. Advanced method for N- and S-phenotype separation

As the changes between the cell types are spontaneous *in vitro*, and the N-type cells become dominant in the parental SH-SY5Y cell culture, it was necessary to develop distinct culturing methods for the N- and S-phenotypes. In the N-type cell culture cells grew in aggregates and

only a few S-type cells were present. In the S-type culture the cells were flat and grew evenly. Already after the second subculture the morphological differences became obvious (Figure 2), though cultures were never completely pure for one phenotype. Dead cells were always present in the cultures, though the viability and proliferation of the living cells was good.

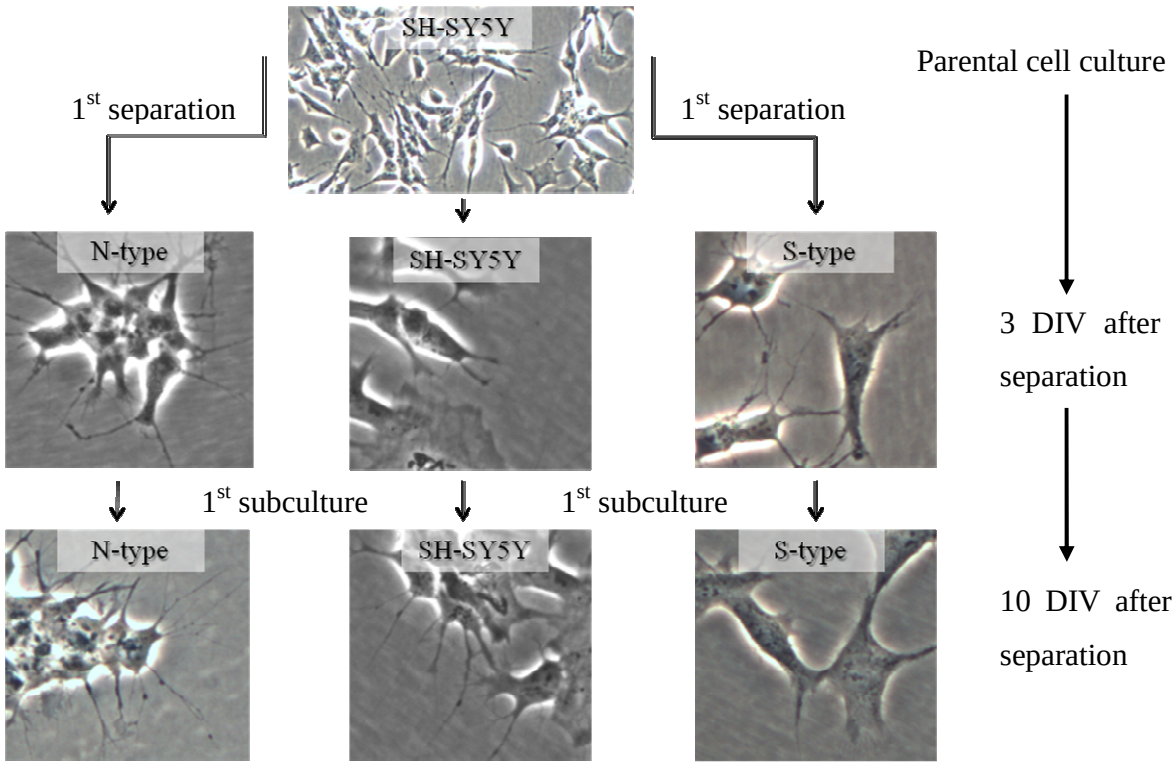

Figure 2. A schematic view of the novel method developed in this study and the succession of the phenotype separation. The differences between the parental SH-SY5Y, N- and S-type cells can clearly be seen after ten days from the initial phenotype separation.

4.3. SH-SY5Y neuroblastoma cell morphology and proliferation

As can be seen in the Figures 3-5, already after two subcultures the N- and S-type cells were clearly distinguishable from each other, and from the parental SH-SY5Y cell line. In Figure 3 the N- and S-type cells are shown by arrows in the parental SH-SY5Y cell culture, though the

differences between the cell types are not as pronounced as in the totally separated N- and S-type cell cultures (Figures 4 and 5). The parental SH-SY5Y cell morphology did not change during normal culturing, though at 8 DIV there was a phase of morphological change, when the cells transiently changed to a more flat phenotype (Figure 3D). Same kind of period showing transient change in morphology was also seen in N- and S-type cells around 8 DIV (Figures 4D and 5D).

The N-type cells grew in aggregates and were smaller than the parental SH-SY5Y cells. The neurites of these cells were short and branching, and the cell somata were triangle-shaped (Figure 4), which is usually considered to be a classical form of neuronal cell soma. Compared to the N-type cells, the S-type cells were flat, large and they did not form aggregates (Figure 5). They also grew thicker and shorter neurites, and after 6 DIV lacked them totally.

For the immunofluorescence experiments antibody against vimentin was chosen to further characterize possible differences between the N- and S-types, because it has earlier been shown to label mainly the S-type cells (Ciccarone et al., 1989; Messi et al., 2008). NF68 was used because of its suggested ability to label the N-type cells (Biagiotti et al., 2006) and Hoechst 33258 label to detect all nuclei in cultures. However, the labels for NF68 and Hoechst 33258 were too faint to be seen in the taken images, and thus are not shown here. Also, NF68 was recently shown by Messi et al. (2008) not to be specific for the N-type cells, and thus was eventually excluded from the experiments.

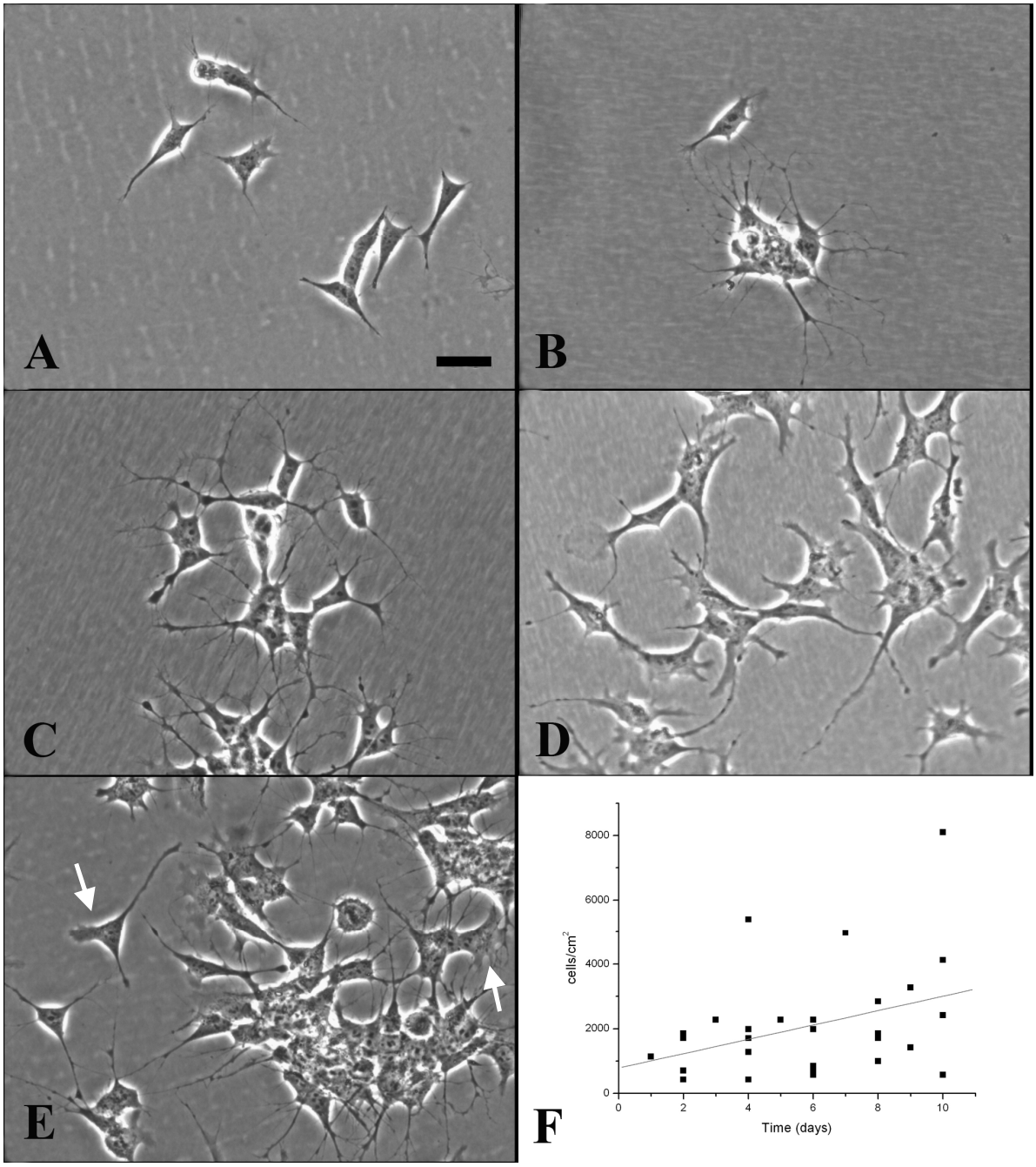

Figure 3. The growth of parental human neuroblastoma SH-SY5Y cell line. In A cells are at 2 DIV, in B at 4 DIV, in C at 6 DIV, and in D at 8 DIV. In E the cells are at 10 DIV and examples of the N- and S-type resembling cells are shown by arrows. In F all the counted data points for the proliferation analysis can be seen with a linear regression (N = 5 in even days and N = 1 in odd days). The scalebar is 20 µm.

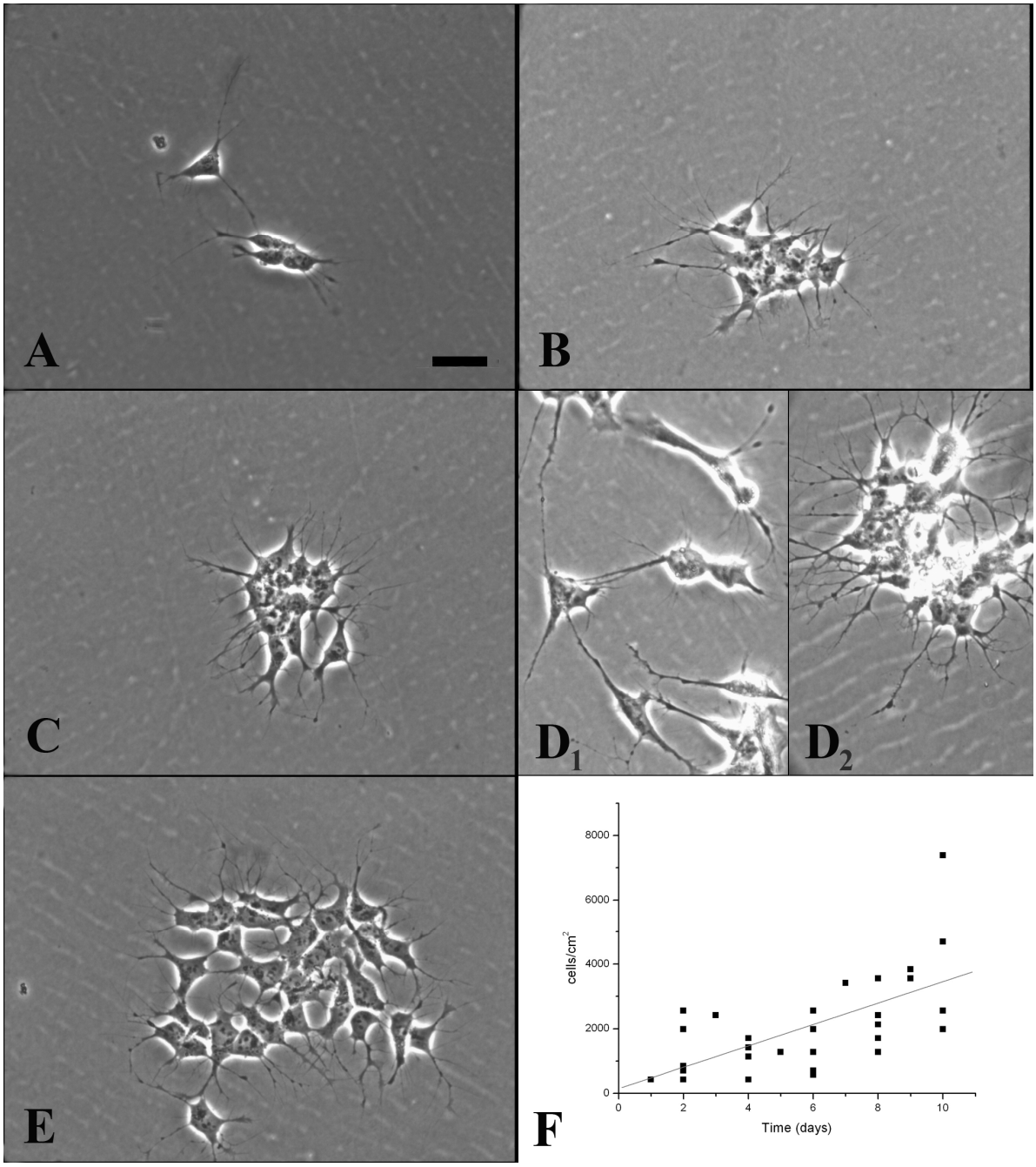

Figure 4. The growth of the N-type cells derived from the parental SH-SY5Y cell line. In A cells are at 2 DIV, in B at 4 DIV, in C at 6 DIV and in D at 8 DIV. Note that the cells seen in D1 are not growing in aggregates. This was seen in about half of the images taken at 8 DIV in all the N-type cell cultures. In E cells are imaged at 10 DIV. In F all the measured data points for the proliferation analysis can be seen with a linear regression (N = 5 in even days and N = 1 in odd days). The scalebar is 20 µm.

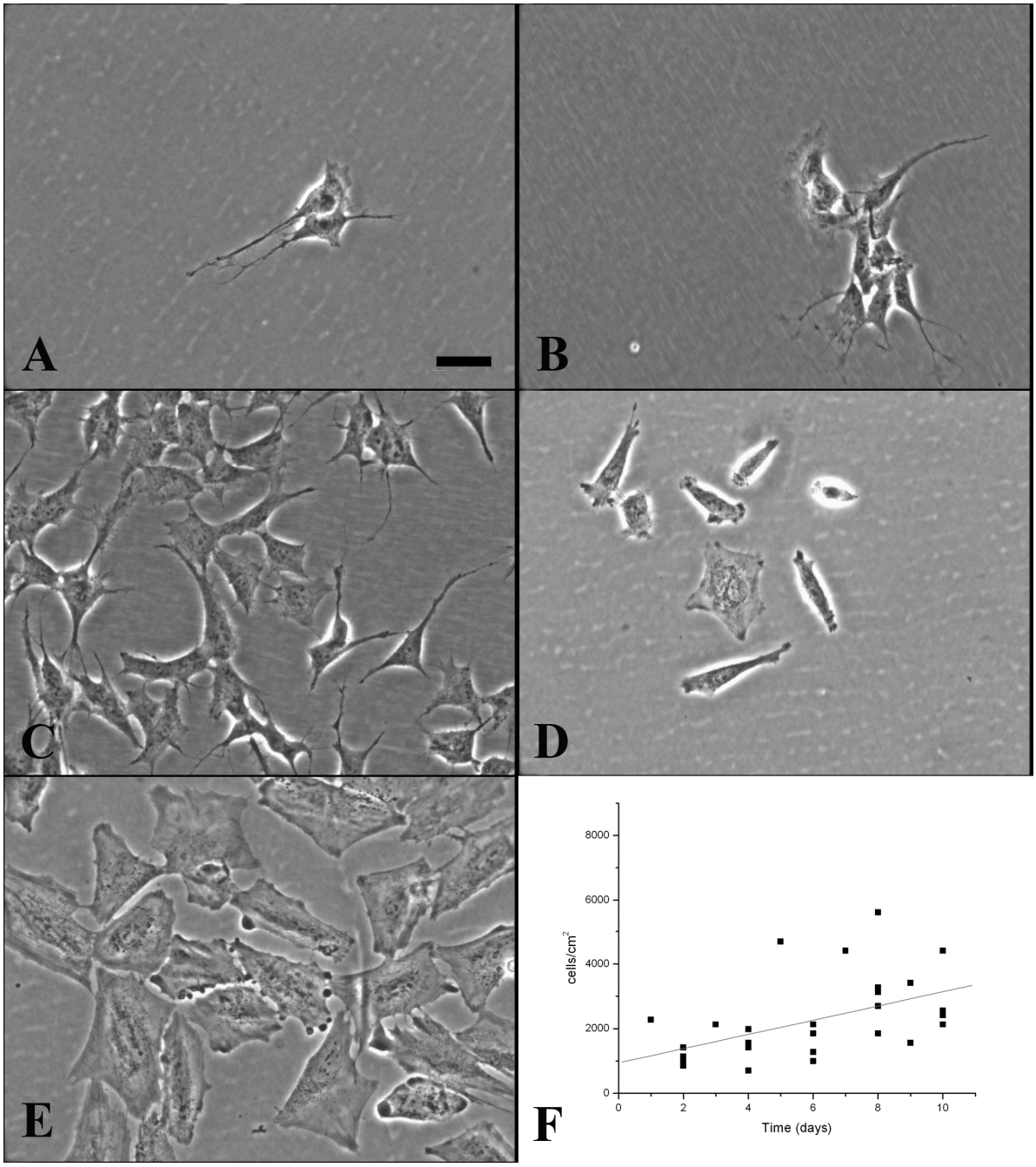

Figure 5. The growth of the S-type cells derived from the parental SH-SY5Y cell line. In A cells are at 2 DIV, in B at 4 DIV, in C at 6 DIV and in D at 8 DIV. The cells on D are distinct from the cells seen at other time points and these different looking S-type cells could be seen in all S-type cell cultures at 8 DIV. In E the cells are imaged at 10 DIV. In F all the measured data points for the proliferation analysis can be seen with a linear regression (N = 5 in even days and N = 1 in odd days). The scalebar is 20 µm.

Vimentin labeled some cells in all cultures, but its fluorescence was clearly more intensive in the S-type cells, than in the N-type cell cultures (Figure 6). There was also a larger number of vimentin-positive cells present in the S-type cell cultures, when compared to the parental SH-SY5Y or N-type cell cultures, and there were no clear neurites in the vimentin-positive cells. The vimentin label was granular in the parental SH-SY5Y cells and smoother in the N-type cells, but otherwise the parental cells and the N-type cells did not differ from each other in the weak vimentin labeling.

Figure 6. The fluorescence images of the vimentin labeled human neuroblastoma cells. In A is the parental SH-SY5Y cell culture, in B is the N-type cell culture and in C is the S-type cell culture. The scalebar is 20 µm.

For the proliferation analysis of the SH-SY5Y cells, the cells were counted in one set of experiments once a day from 1 DIV to 8 DIV and on other four sets of experiments every second day from 2 DIV to 10 DIV. These results are shown in Table I. The linear regressions related to this analysis can be found in Figures 3-5F. It can be seen from Table I and the graphs shown in Figures 3-5, that the proliferation of the parental SH-SY5Y, and the N- and S-type cells followed approximately same pattern. The number of the parental SH-SY5Y cells increased over the time, but large variations with random increase and decrease in the cell number was seen. The N-type cells showed same kind of variation, and continued proliferation at 10 DIV, which may indicate, that these cells could be cultured for longer times. When compared to the parental SH-SY5Y or N-type cells, the S-type cells proliferate slower towards 10 DIV. The significant differences marked with stars in Table I and Table II (page 38) describe the differences between the non-treated control cells and RA, as well as between controls and RA + chol treated cells, and will be described in more detail in the next section 4.4.

Table I. The means and standard errors (SE) of the SH-SY5Y cell numbers in the controls (cells without any treatments). ** marks significant differences ($P < 0.05$) between the control and RA, and between the control and RA + chol treatments in both the N- and S-type cells (see also Table II, page 38). N = 5 for each cell type at each time point.

2 DIV

Cell line	Mean ± SE
SH-SY5Y	1079.2 ± 289.6
N-type	1306.4 ± 410.1
S-type	1100.5 ± 121.2

4 DIV

Cell line	Mean ± SE
SH-SY5Y	2158.6 ± 851.5
N-type	1164.4 ± 212.5 **
S-type	1420.0 ± 205.8 **

6 DIV

Cell line	Mean ± SE
SH-SY5Y	1278.0 ± 353.6
N-type	1420.0 ± 378.4
S-type	1448.4 ± 230.7

8 DIV

Cell line	Mean ± SE
SH-SY5Y	1817.6 ± 295.8
N-type	2215.4 ± 385.4
S-type	3311.2 ± 628.1

10 DIV

Cell line	Mean ± SE
SH-SY5Y	3799.2 ± 1605.3
N-type	4154.0 ± 1223.9 **
S-type	2875.7 ± 516.7 **

4.4. Effects of retinoic acid and cholesterol on SH-SY5Y cells

All SH-SY5Y cell cultures were treated with retinoic acid to follow their ability to differentiate and with cholesterol to asses its effects on cell phenotypes.

4.4.1. Morphology of SH-SY5Y cells

The parental SH-SY5Y cells treated with RA developed longer neurites (Figure 7), when compared to the neurites of non-treated controls (Figure 3), and the cell somata appeared to be triangle-like (a classical neuron). In the parental SH-SY5Y cells no clear morphological differences were seen between the cells treated with RA or cholesterol (Figure 7), though cells

treated with RA looked slightly more like the N-type cells when compared to treatment with cholesterol. With cholesterol treatment the neurites of the cells were short until around 10 DIV, when extremely long neurites could be detected. With cholesterol and RA + chol treatments there were S-type cells present in the parental SH-SY5Y cultures (Figure 7H and I_2). With RA + chol treatment the neurites seemed to be shorter and thicker than what was seen with cells treated with only RA. Cholesterol caused increased amounts of cell debris in all cell cultures; more when only cholesterol was present and less when also RA was present.

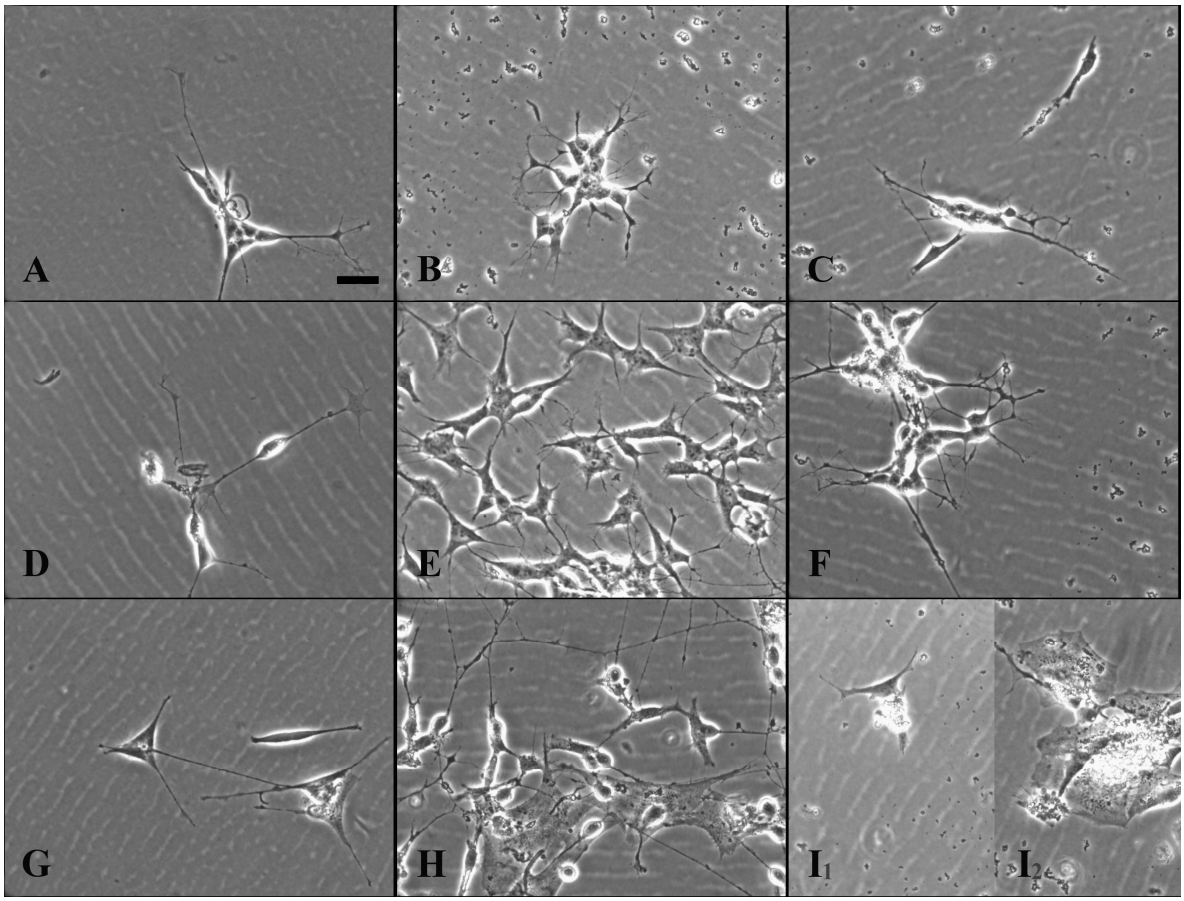

Figure 7. Retinoic acid and cholesterol treatments on the parental SH-SY5Y cells. The treatments are in vertical columns and the time points are in horizontal columns. In A, D and G are RA treatments at 4, 7 and 10 DIV, respectively, in B, E and H are cholesterol treatments at 4, 7 and 10 DIV, respectively, and in C, F and I are RA + chol treatments at 4, 7 and 10 DIV, respectively. The scalebar is 20 µm.

In the RA treated N-type cells the neurites grew longer and cell somata became slightly smaller (Figure 8), when compared to the non-treated controls (Figure 4). When treated with cholesterol, the neurites of the N-type cells were shorter than in the RA treated cells, and the cells appeared to be similar to the non-treated control cells. The cells grew in colonies as in controls, but after 8 DIV the cells treated with cholesterol grew more evenly. The N-type cells treated with RA + chol also had longer neurites than what was seen in the non-treated controls. The slight apoptotic effect of RA treatment could be seen at 10 DIV, as there were dead N-type cells and live S-type cells in the cultures (Figure 8I).

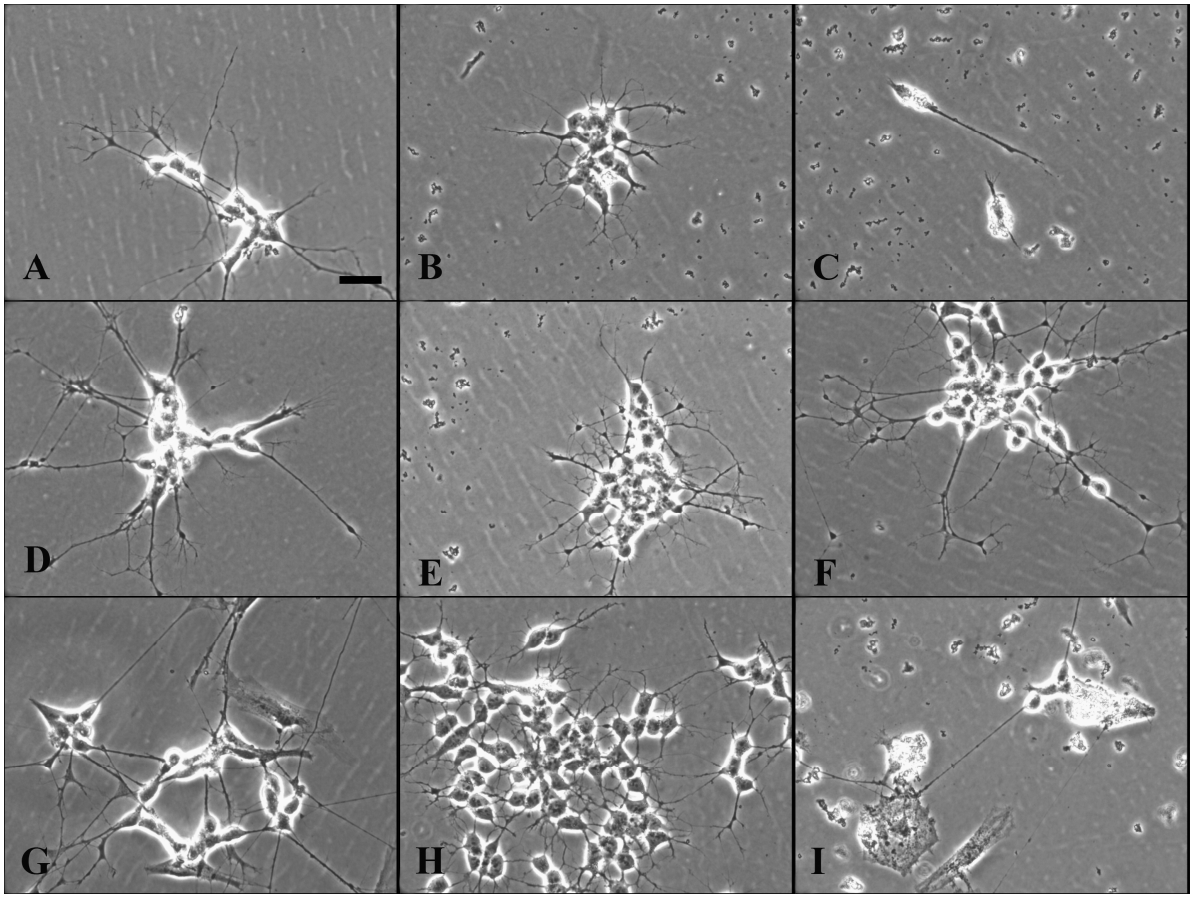

Figure 8. Retinoic acid and cholesterol treatments on the N-type cells where the treatments are in vertical columns and the time points are in horizontal columns. In A, D and G are RA treatments at 4, 7 and 10 DIV, respectively, in B, E and H are cholesterol treatments at 4, 7 and 10 DIV, respectively, and in C, F and I are RA + chol treatments at 4, 7 and 10 DIV, respectively. The scalebar is 20 µm.

The S-type cells treated with RA did not change their morphology. When the S-type cells were treated with cholesterol for 7 DIV the cells developed a very different morphology (Figure 9E) compared to all the other samples in Figure 9. The same kind of transient reversion to the N-type-like morphology was observed also in the S-type control cells around 6 DIV (Figure 5C). However, most of those smaller, process-bearing cells disappeared when cells were further cultured.

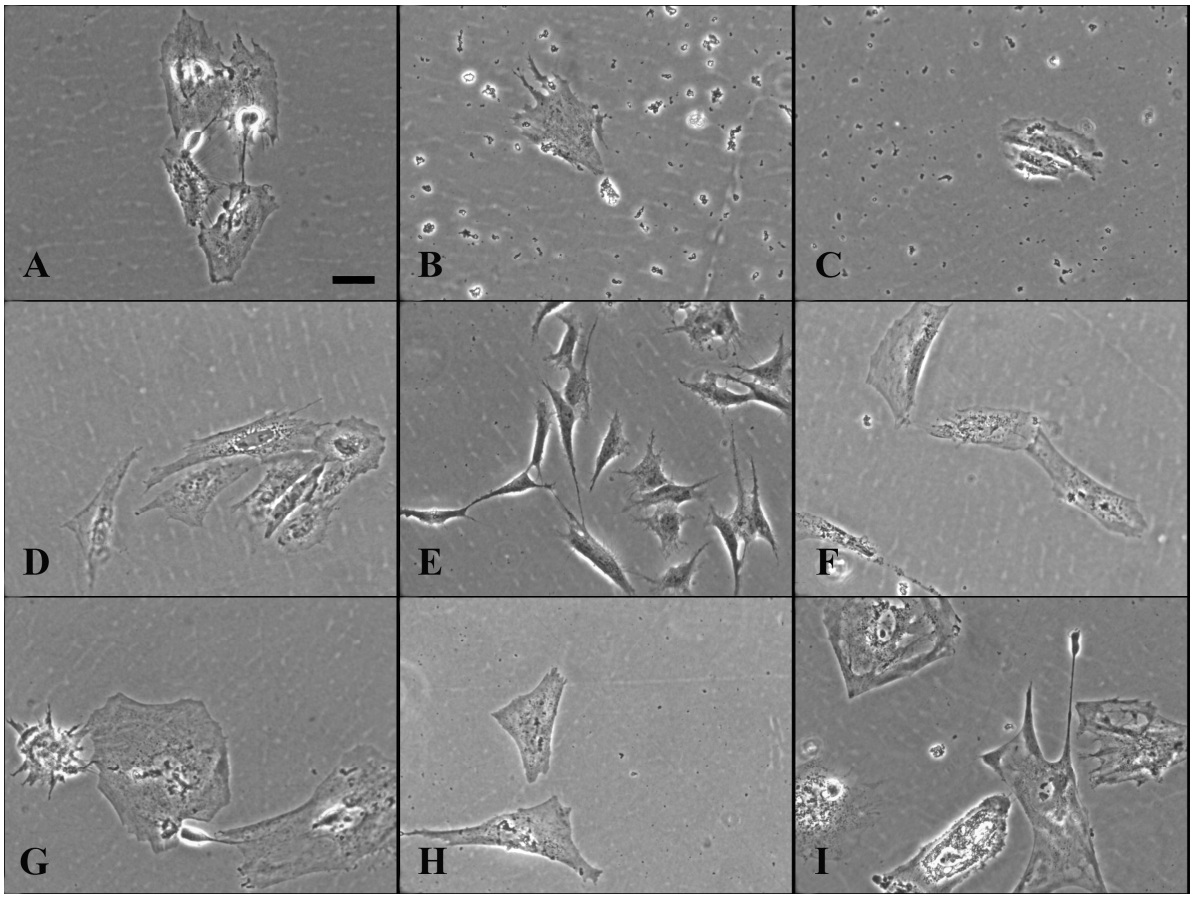

Figure 9. Retinoic acid and cholesterol treatments on the S-type cells, where the treatments are in vertical columns and the time points are in horizontal columns. In A, D and G are RA treatments at 4, 7 and 10 DIV, respectively, in B, E and H are cholesterol treatments at 4, 7 and 10 DIV, respectively. The distinct cell morphology seen in E was observed in most of the images taken at that time point in all sets of S-type cell cultures. In C, F and I RA + chol treatments are shown at 4, 7 and 10 DIV, respectively. The scalebar is 20 µm.

Vimentin fluorescence labeling was strong in all SH-SY5Y cell cultures with no big differences in fluorescence intensity between RA and cholesterol treatments. However, only some of the cells in each field were vimentin positive and only these stained cells are seen in Figure 10. In especially the parental and S-type cultures vimentin labeled cells with neurites, which was not seen in non-treated controls (Figure 6). The label seemed to be grainy at the borders of the cells treated with RA, when compared to the images taken of cholesterol or RA + chol treated cells (Figure 10).

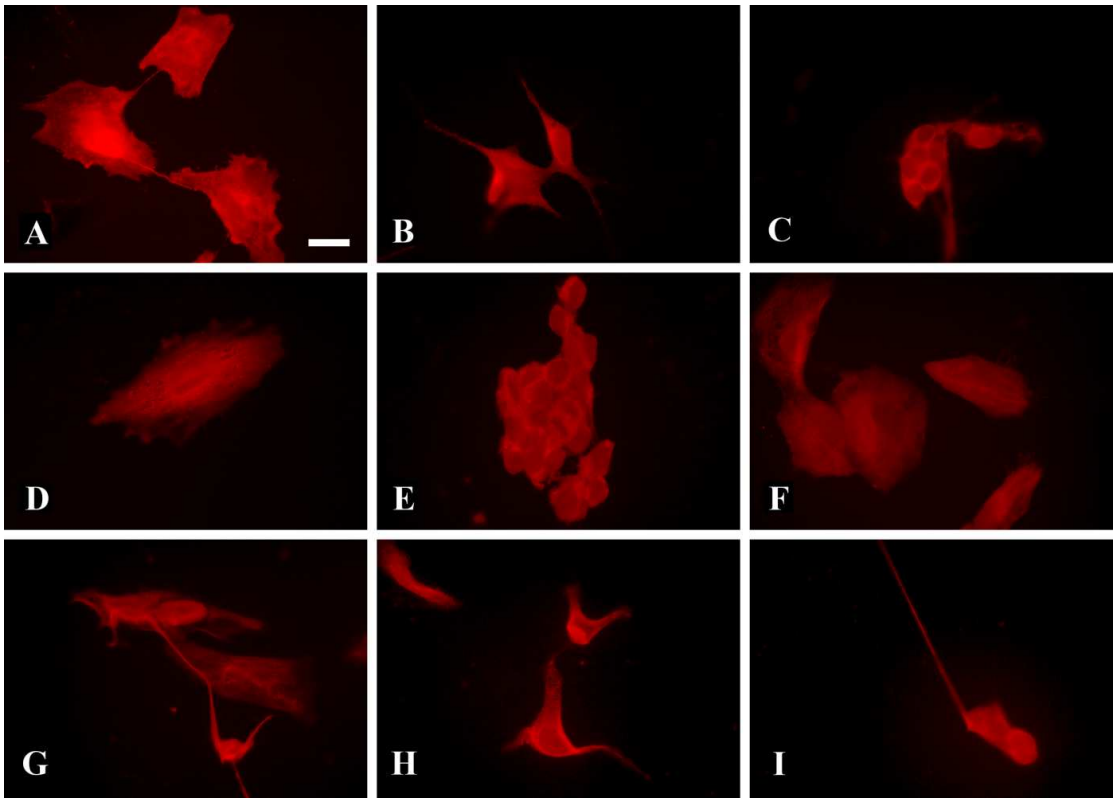

Figure 10. Vimentin fluorescence in the parental SH-SY5Y cells (A-C), in the N-type (D-F) and in the S-type (G-I) cells, where A, D and G are RA, B, E and H are cholesterol and C, F and I are RA + chol treatments. All the cells were cultured for ten days. The scalebar is 20 µm.

4.4.2. Proliferation of retinoic acid and cholesterol treated SH-SY5Y cells

The general trend in the cell numbers of the control, RA and cholesterol treated human neuroblastoma cells can be seen in Figure 11. There was a large variation in the number of cells in all treatments. When non-treated, all cell types had similar proliferation pattern, except the parental SH-SY5Y cells at 2 DIV and the S-type cells at 8 DIV. However, there were no statistically significant differences ($P > 0.05$) between the non-treated control cultures. The parental SH-SY5Y, N- and S-type cells had similar proliferation patterns also when treated with RA and cholesterol.

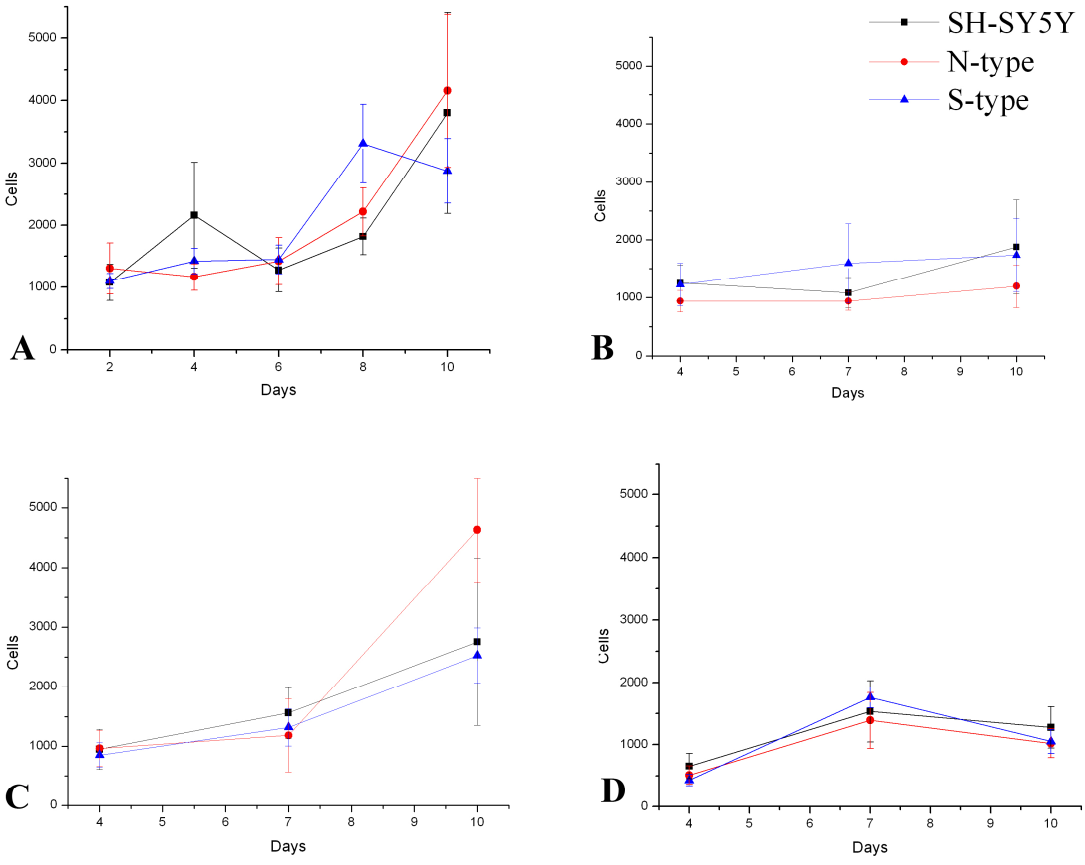

Figure 11. Cell proliferation of the parental SH-SY5Y, N-type and S-type cells, where A is control, B is RA treated cells, C is cholesterol treated cells and D is RA + chol treated cells.

During the ten days of culturing, there was no change in cell proliferation in the parental SH-SY5Y, N- or S-type cells treated with RA (Table II). When the non-treated control N-type cell cultures (Table I) were compared to the N-type cell cultures treated with RA (Table II), there was a significant difference ($P = 0.037$) in cell proliferation at 10 DIV. When the RA and cholesterol treated N-type cell cultures were compared, there was a significant difference ($P = 0.007$) in cell proliferation at 10 DIV. In both of these the amount of cells was less in those cultures, where RA was present. There were no statistically significant differences ($P > 0.05$) between the cell numbers in the RA or RA + chol treated N- and S-type cell cultures or between these and the parental SH-SY5Y cell cultures.

With cholesterol treatments, there was a slight reduction in the number of cells when compared to non-treated control cell cultures, and there was also a significant difference ($P = 0.019$) between the N- and S-type cell cultures at 10 DIV, where there were less cells in the N-type cell cultures. Between cholesterol and RA + chol treated N- and S-type cell cultures there were significant differences ($P = 0.004$ and 0.018, respectively) in the treatments at 10 DIV, where there were less cells in the RA + chol treated cell cultures.

With RA + chol treatment the cell proliferation of the parental SH-SY5Y, N- and S-type cell cultures were reduced after an initial growth (Table II and Figure 11D). Also, when the control cells were compared to RA + chol treated N- and S-type cell cultures, there were significant differences at 4 DIV ($P = 0.035$ and 0.002, respectively) and at 10 DIV ($P = 0.025$ and 0.008, respectively), where there were less cells in the RA + chol treated cell cultures.

Table II. Means and standard errors in the number of the RA, cholesterol and RA + chol treated human neuroblastoma cells. ** marks significant differences ($P < 0.05$) between control (see also Table I) and RA, as well as between control and RA + chol treatments in both the N- and S-type cells. Significant difference could also be seen in cholesterol treatment between the N- and S-type cells, between RA and cholesterol treatments in the N-type cells, and between cholesterol and RA + chol treatment in the N-type cells. Also, in the S-type cells there was a significant difference between cholesterol and RA + chol treatments. N = 5 for each cell type in each time point.

RA

4 DIV	Mean ± SE
SH-SY5Y	1249.6 ± 312.4
N-type	937.2 ± 182.9
S-type	1221.2 ± 366.5

7 DIV	Mean ± SE
SH-SY5Y	1079.0 ± 262.5
N-type	937.2 ± 152.9
S-type	1590.6 ± 687.7

10 DIV	Mean ± SE
SH-SY5Y	1874.6 ± 813.9
N-type	1192.8 ± 369.2 **
S-type	1732.6 ± 631.1

Cholesterol

4 DIV	Mean ± SE
SH-SY5Y	948.8 ± 339.6
N-type	965.6 ± 302.6
S-type	852.0 ± 209.0

7 DIV	Mean ± SE
SH-SY5Y	1562.0 ± 426.0
N-type	1185.0 ± 620.6
S-type	1320.0 ± 314.5

10 DIV	Mean ± SE
SH-SY5Y	2755.2 ± 1400.1
N-type	4630.2 ± 875.2 **
S-type	2528.2 ± 459.1 **

RA + chol

4 DIV	Mean ± SE
SH-SY5Y	653.2 ± 203.8
N-type	511.2 ± 146.2 **
S-type	426.2 ± 89.8 **

7 DIV	Mean ± SE
SH-SY5Y	1533.8 ± 494.9
N-type	1390.2 ± 450.4
S-type	1760.8 ± 165.6

10 DIV	Mean ± SE
SH-SY5Y	1278.0 ± 336.0
N-type	1022.4 ± 235.0 **
S-type	1050.8 ± 193.7 **

4.5. U373-MG astrocytoma cell morphology and proliferation

The human astrocytoma U373-MG cell line was selected to be used as a comparison to the neuroblastoma SH-SY5Y cell line. Same morphology and proliferation experiments were carried out with both tumor cell lines.

4.5.1. Morphology of U373-MG cells

The morphology images of the human astrocytoma U373-MG cells are shown in Figure 12 where it can be seen, that the cells became flatter and bigger and the amount of cells increased during culturing, but the cells never reached confluency in these culture conditions.

In this study in addition to vimentin, the antibody against glial fibrillary acidic protein was chosen for cell characterization. GFAP has been shown to be a marker of reactive type of astrocytes and neurotoxicity (Ciccarone et al., 1989; Toimela et al., 1995; Encinas et al., 2000). As is seen in Figure 13, the cell nuclei were only weakly stained, and GFAP label was faintly detectable around the nucleus. Vimentin was distributed throughout the cytoplasm, being especially strong around the nucleus.

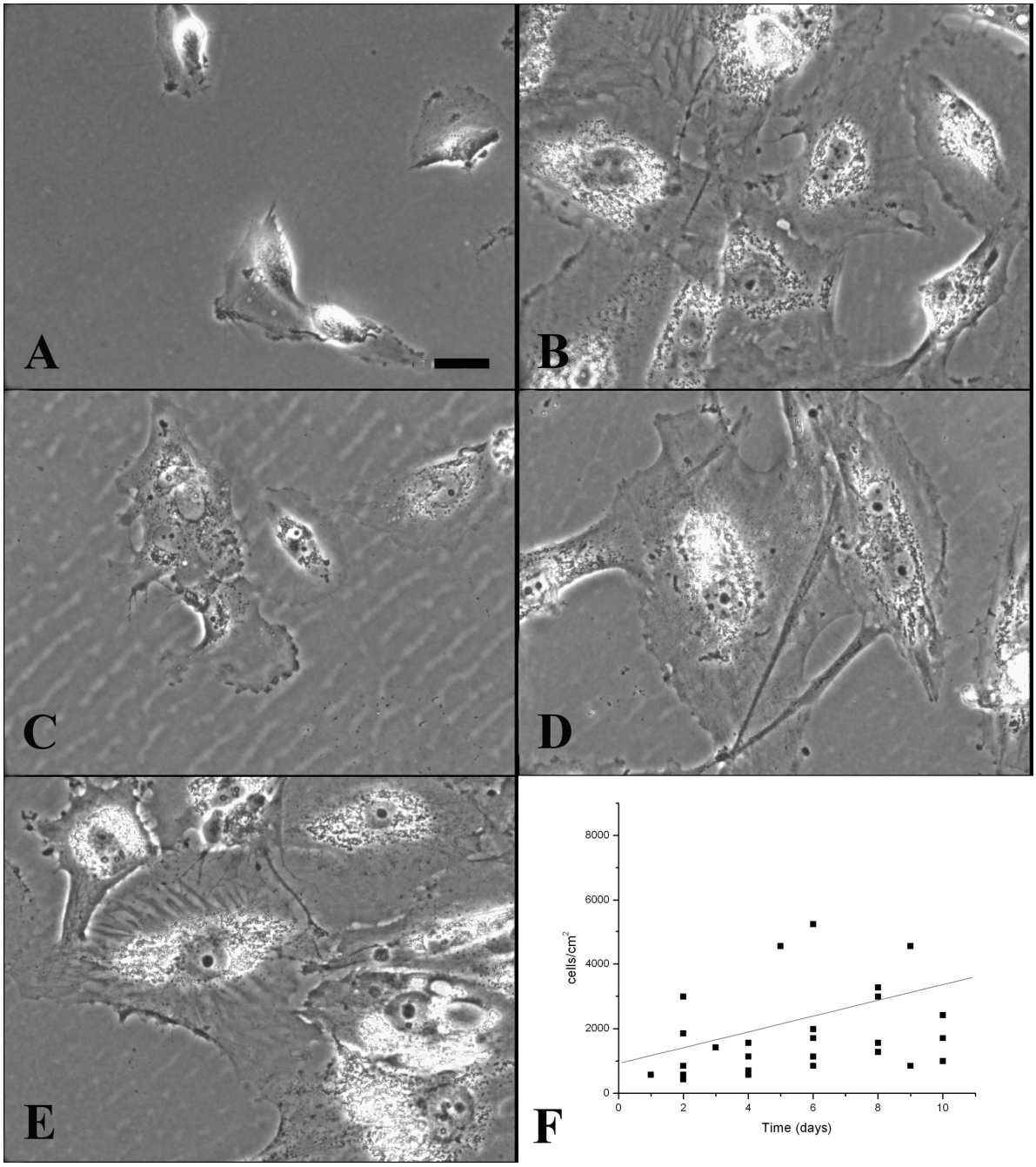

Figure 12. The growth of the human astrocytoma U373-MG cell line. In A cells are at 2 DIV, in B at 4 DIV, in C at 6 DIV, in D at 8 DIV and in E at 10 DIV. In F are the measured data points for the proliferation analysis with a linear regression (N = 5 in even days and N = 1 in odd days). One data point (11659 at 8 DIV) was excluded to get uniform scale for all the graphs shown in SH-SY5Y and U373-MG cell morphology Figures 3-5F and 12F. The scalebar is 20 µm and the stripes seen in the images are artifacts from the used Falcon dishes.

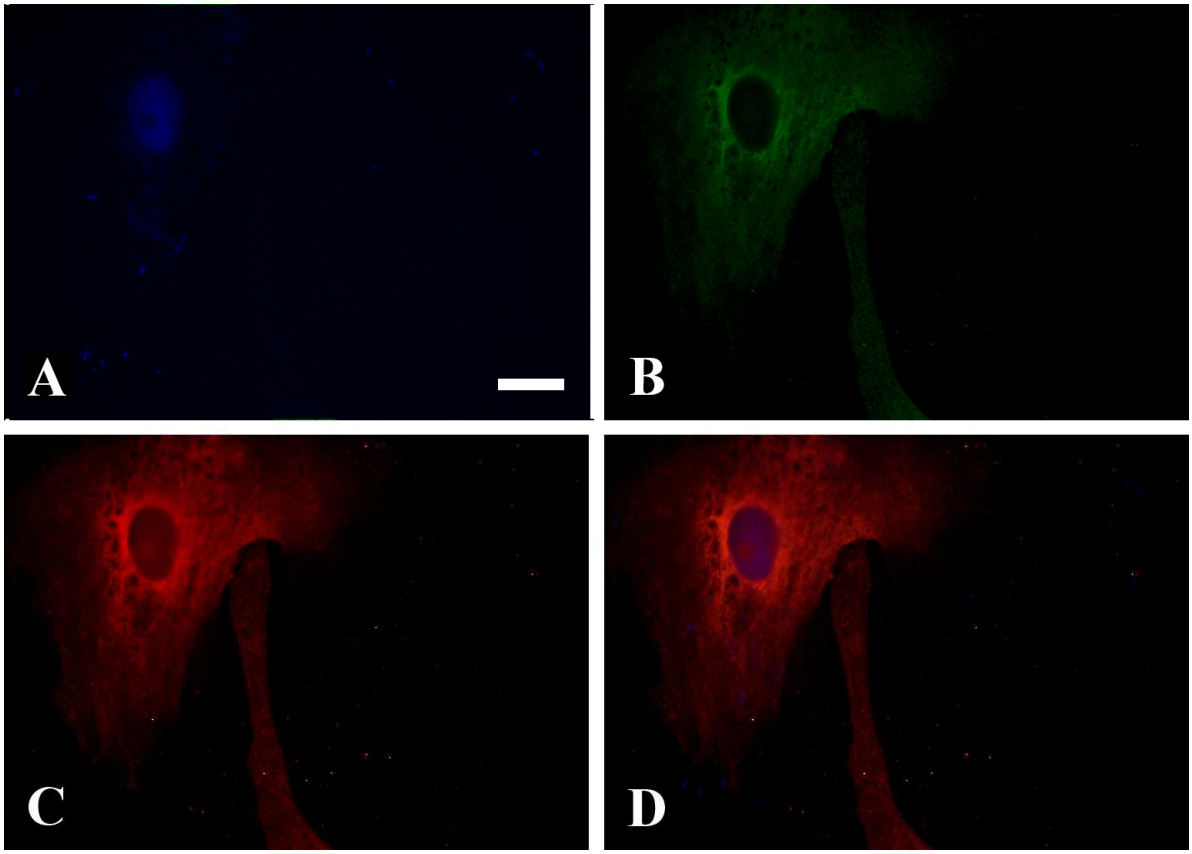

Figure 13. The human astrocytoma U373-MG cell where A is nucleus stained with Hoechst 33258, B is GFAP label, C is vimentin label and D is the merged image. The scalebar is 20 µm.

4.5.2. Retinoic acid and cholesterol treatments of U373-MG cells

When the human U373-MG astrocytoma cells were treated with RA, their morphology changed to a flatter form (Figure 14) when compared to non-treated control cells (Figure 12). When treated with cholesterol, cells developed long processes which were not seen in controls. The long processes were also seen when the cells were treated with RA + chol, and thus may partly be cholesterol induced.

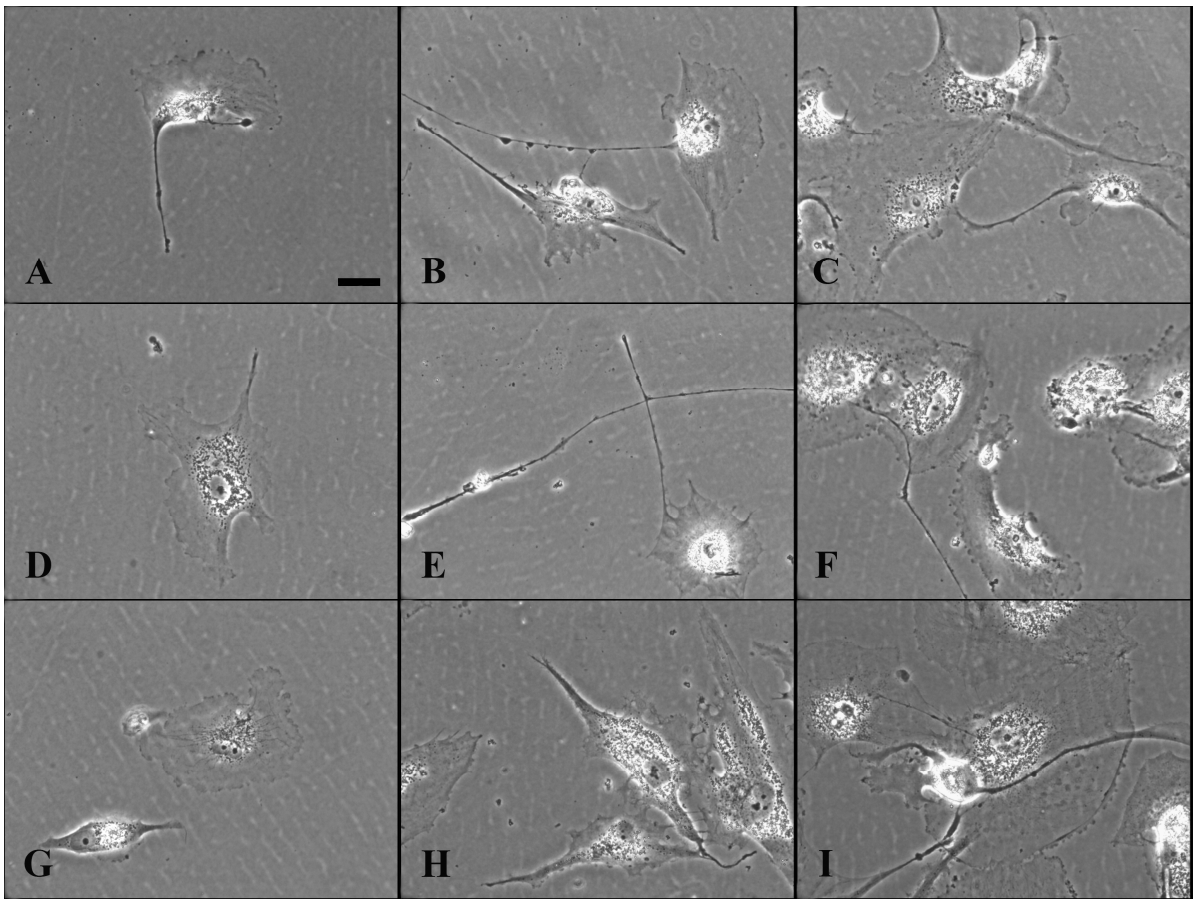

Figure 14. Retinoic acid and cholesterol treatments of the human astrocytoma U373-MG cells, where the treatments are in vertical columns and the time points are in horizontal columns. In A, D and G are RA treatments at 4, 7 and 10 DIV, respectively, B, E and H are cholesterol treatments at 4, 7 and 10 DIV, respectively, and C, F and I are RA + chol treatments at 4, 7 and 10 DIV, respectively. The scalebar is 20 µm.

From the vimentin and GFAP fluorescence images (Figure 15) it can be seen, that the human astrocytoma cells treated with RA did not survive well. When compared to Figure 13, the GFAP labeling was much stronger with these treatments than in non-treated control cells. When treated with cholesterol, the U373-MG cells appeared to be normal in morphology, but when RA was present (the RA + chol treatments), the cells shrunk and eventually died.

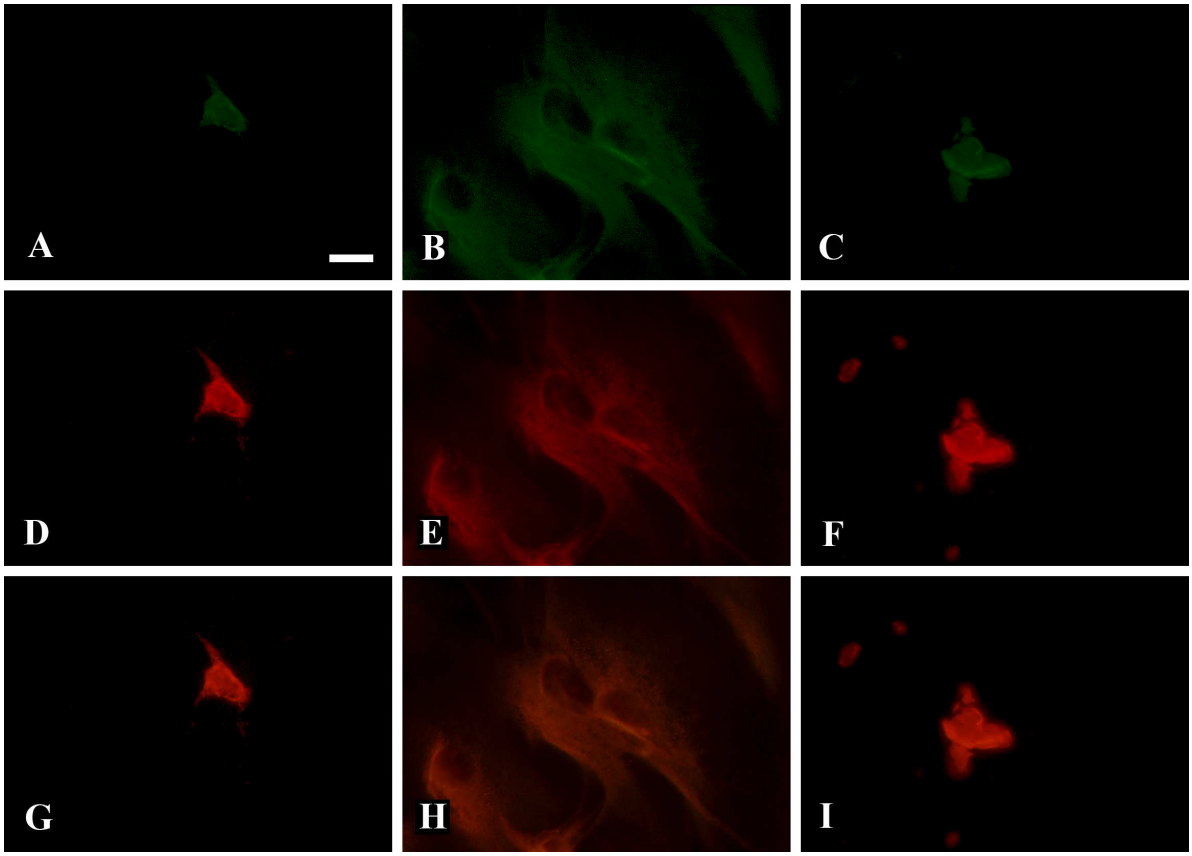

Figure 15. Fluorescence pictures of the U373-MG cells, where A-C is GFAP, D-F is vimentin and G-I are merged images. In A, D and G are RA treated cells, in B, E and H are cholesterol treated cells and in C, F and I are RA + chol treated cells at 10 DIV. Scalebar is 20 µm.

4.5.3. Proliferation of U373-MG cells

The linear regression related to the U373-MG proliferation analysis can be found in Figure 12F. In one set of experiments the cells were counted once a day from 1 DIV to 8 DIV, and on other four sets of experiments every second day from 2 DIV to 10 DIV. The results of the cell counts can be seen in Table III. The general trend in the cell numbers of the control, RA and cholesterol treated U373-MG cells are seen in Figure 16. After the initial growth, the number of cells was generally lower at 4 DIV, and again decreased after 8 DIV. This behavior was seen in all the U373-MG cell cultures. As with the human neuroblastoma cell cultures, the U373-MG cells showed random increase and decrease in the cell numbers in the non-treated

control cell cultures. With RA alone the cells had no change in proliferation, and with cholesterol treatments the numbers of cells increase slightly after 7 DIV. In RA + chol treatments the cell number slightly increases until 7 DIV.

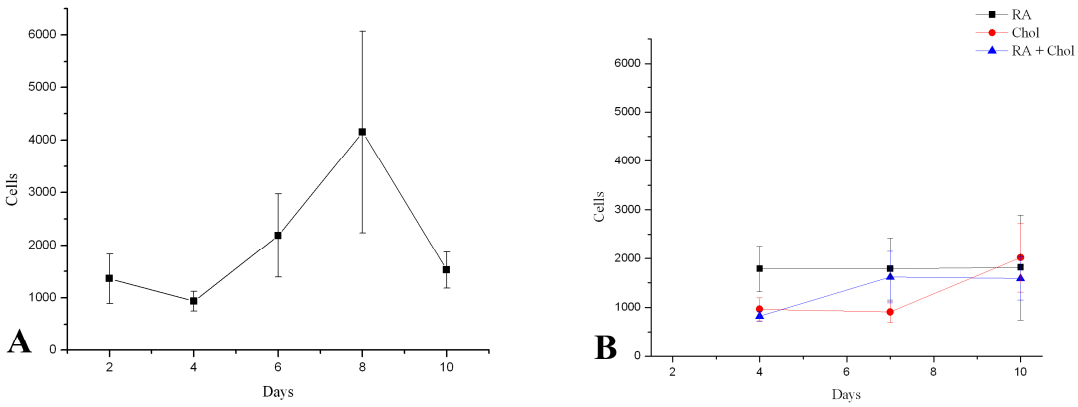

Figure 16. Cell proliferation of the U373-MG cells where A is control and B is cells treated with RA and cholesterol.

When the number of cells was counted in RA, cholesterol and RA + chol treated U373-MG cell cultures, no statistically significant differences ($P > 0.05$) were detected, though as shown in Figure 16, cells grew better in the non-treated control cultures. After 8 DIV, the cell numbers appeared to be almost the same in all treatments with RA and cholesterol.

Table III. Means and standard errors in the number of human astrocytoma cells in controls, and in cells treated with RA and cholesterol. N= 5 in each time point.

control

DIV	Mean ± SE
2	1363.2 ± 470.1
4	937.2 ± 182.9
6	2187.0 ± 792.8
8	4149.6 ± 1916.7
10	1526.6 ± 339.9

RA

DIV	Mean ± SE
4	1789.4 ± 463.8
7	1789.4 ± 637.8
10	1817.8 ± 1076.4

cholesterol

DIV	Mean ± SE
4	965.6 ± 235.0
7	908.8 ± 213.5
10	2016.6 ± 699.3

RA + chol

DIV	Mean ± SE
4	823.6 ± 104.3
7	1619.0 ± 525.0
10	1590.6 ± 438.8

4.6. Effects of manganese on SH-SY5Y and U373-MG cells

The neuroblastoma SH-SY5Y and astrocytoma U373-MG cells were treated with manganese to find out if these cells have distinctly different responses to the manganese-induced apoptosis. Also treatments with manganese together with RA and cholesterol were introduced. Manganese notably reduced the amount of cells, as seen in Figure 17, where the nuclei of the parental SH-SY5Y cells are shown. The same pattern could be seen in all neuroblastoma and astrocytoma cell cultures.

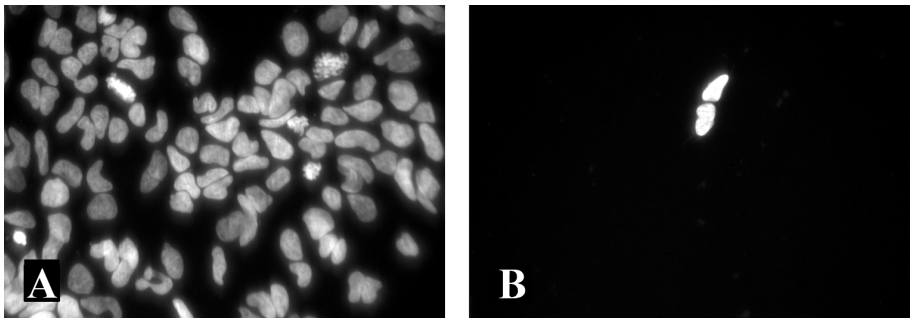

Figure 17. Hoechst 33258 labeled cell nuclei of the parental SH-SY5Y cells. In A are the non-treated control cells and in B are the manganese treated cells. It can clearly be seen, that the amount of cells is markedly reduced.

4.6.1. Effects of manganese on SH-SY5Y cells

The SH-SY5Y cells were grown for 10 DIV and were either non-treated or treated with RA, cholesterol or RA + chol before adding manganese. The results of the manganese treatments in the neuroblastoma cells are presented in Figures 18 and 19, and in Table IV. The N-type cells seemed to be most sensitive to manganese, and the S-type cells the most resistant ones, though no statistically significant differences ($P > 0.05$) were seen. In the parental SH-SY5Y and N-type cell cultures mostly S-type cells were present after manganese-induced apoptosis. With manganese alone and with manganese and cholesterol treatments, the cells survived better than with manganese together with RA or RA + chol treatments.

As before, mostly the S-type-like cells were positive for vimentin (Figure 19). Vimentin staining was also more intense in manganese treated cells than in non-treated control cells (Figure 6).

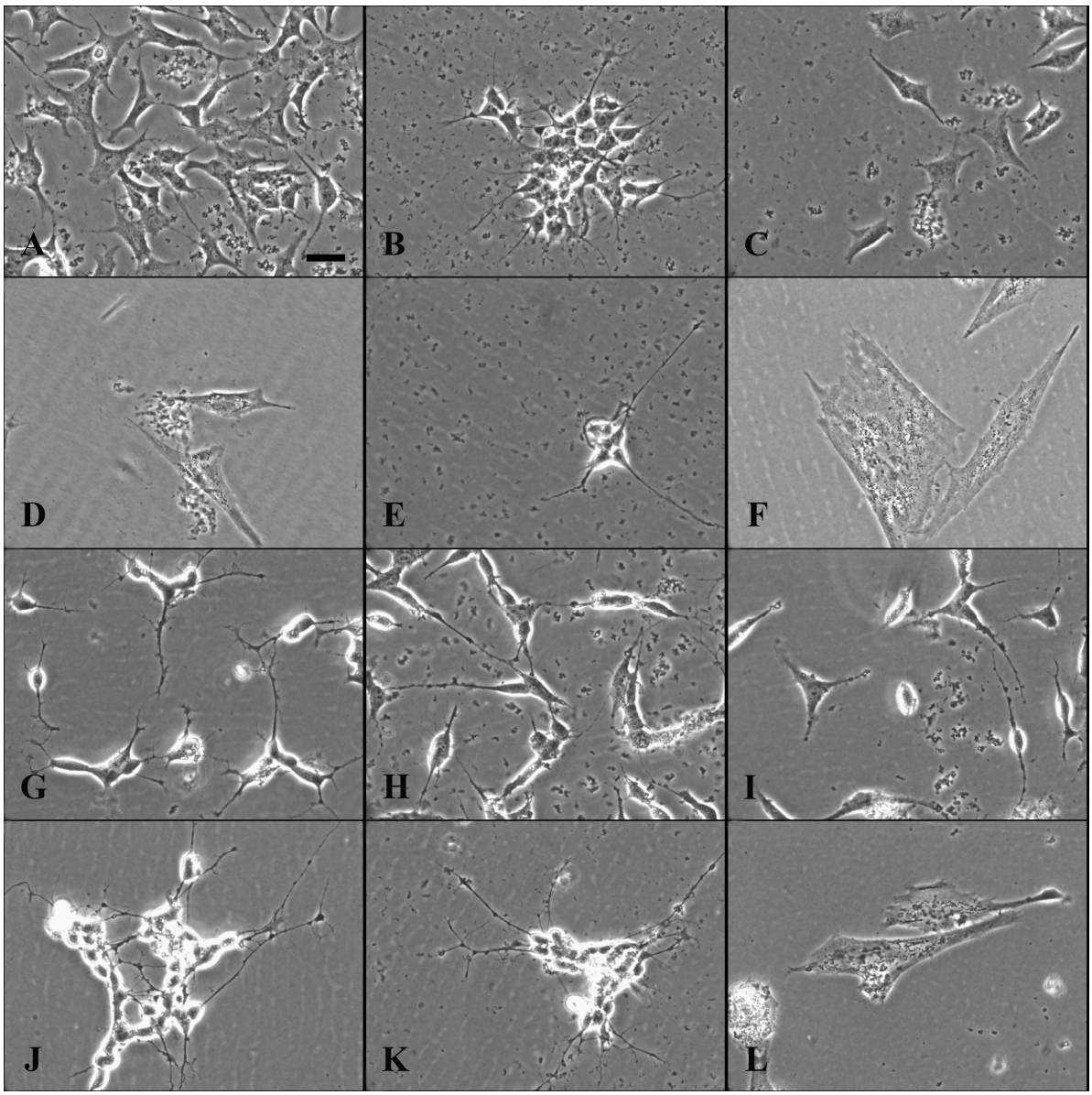

Figure 18. Manganese treatments of the human neuroblatoma cells at 10 DIV, where the treatments are in horizontal columns and the cell types in vertical columns. The parental SH-SY5Y cells are in A, D, G and J, the N-type cells are in B, E H and K, and the S-type cells are in C, F, I and L, where A, B and C are manganese treatments, D, E and F are manganese with RA treatments, G, H and I are manganese with cholesterol treatments and J, K and L are manganese with RA + chol treatments. The scalebar is 20 µm.

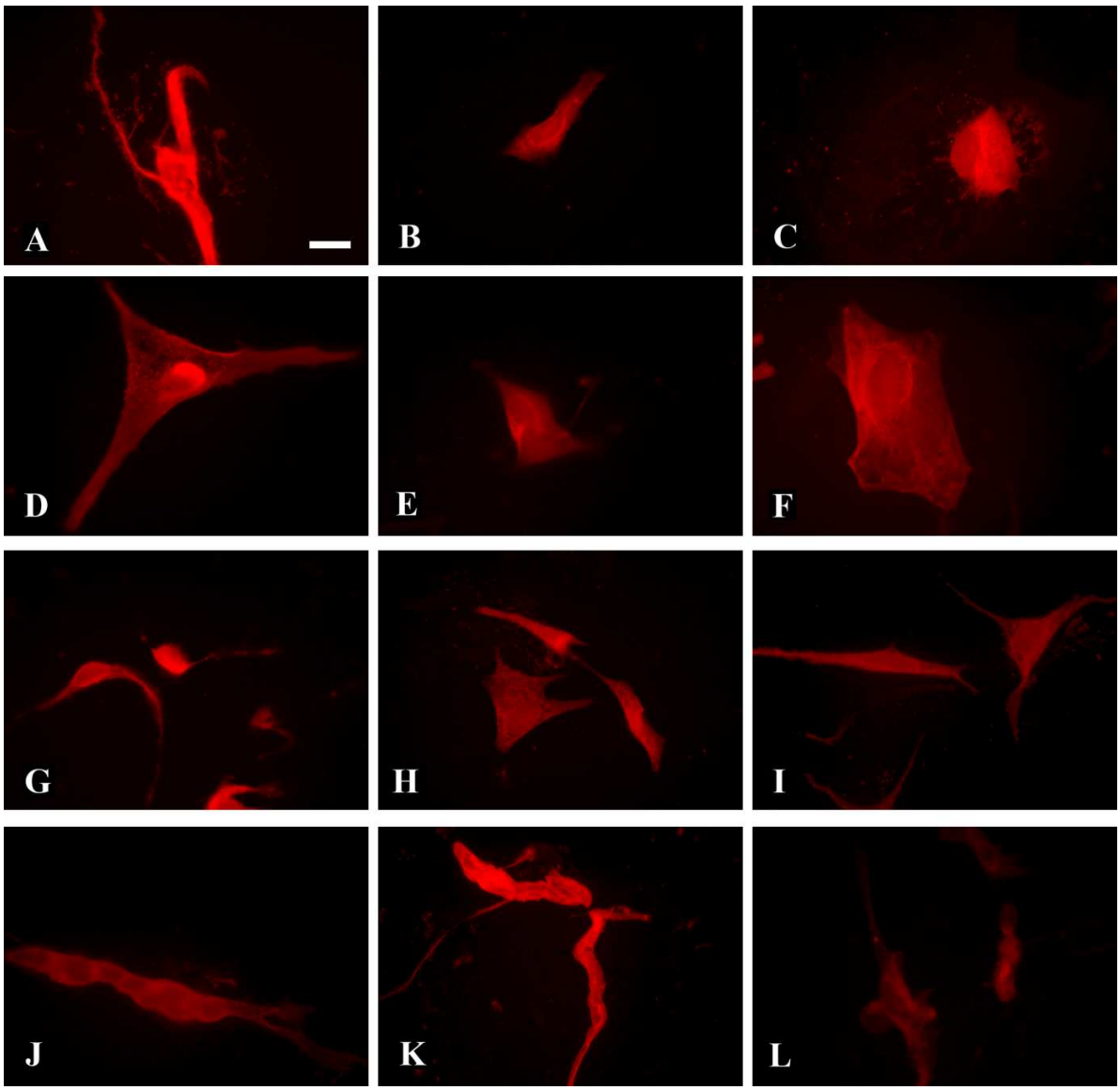

Figure 19. Vimentin fluorescence of the parental SH-SY5Y (A, D, G and J), of the N-type (B, E, H and K) and of the S-type (C, F, and L) cells, where A-C are manganese, D-F manganese with RA, G-I manganese with cholesterol and J-L manganese with RA + chol treatments at 10 DIV. The scalebar is 20 µm.

There was a significant difference (P = 0.002) between the parental SH-SY5Y and the S-type cells in their sensitivity to manganese treatments, when more cells survived in the S-type cell cultures. Significant differences could be found in the parental SH-SY5Y cell numbers between manganese and manganese with RA treatments, and between manganese and manganese with RA + chol treatments (P = 0.019 and 0.003, respectively). In the N- type

cells, there were statistically significant differences between manganese with RA and manganese with cholesterol treatments, and between manganese with cholesterol and manganese with RA + chol treatments (P = 0.042 and 0.022, respectively). In the S-type cells there were significant differences between manganese and manganese with RA treatments, and between manganese and manganese with RA + chol treatments (P = 0.0001 and 0.025, respectively), similarly to the differences in the parental SH-SY5Y cells. In all these there were fewer cells in manganese with RA or manganese with RA + chol treatments than in the manganese or manganese with cholesterol treated cell cultures.

Table IV. Means and standard errors in different types of SH-SY5Y cells in manganese, manganese with RA, manganese with cholesterol and manganese with RA + chol treatments. ** marks significant differences between the manganese treated parental SH-SY5Y and N-type cells, between manganese and manganese with RA, and between manganese and manganese with RA + chol treatments in the parental SH-SY5Y cells. . In the N-type cells significant differences could be seen between manganese with RA and manganese with chol, and between manganese with chol and manganese with RA + chol treatments. Significant differences could be seen also between manganese and manganese with RA, and manganese with RA + chol treatments in the S-type cells (N = 3 per cell type in each treatment).

Mn

Cell type	Mean ± SE
SH-SY5Y	994.0 ± 82.0 **
N-type	1941.0 ± 593.4
S-type	1562.0 ± 0.0 **

Mn + RA

Cell type	Mean ± SE
SH-SY5Y	520.7 ± 94.7 **
N-type	473.3 ± 125.2 **
S-type	284.0 ± 82.0 **

Mn + cholesterol

Cell type	Mean ± SE
SH-SY5Y	1136.0 ± 375.7
N-type	1420.0 ± 295.6 **
S-type	994.0 ± 409.9

Mn + RA + chol

Cell type	Mean ± SE
SH-SY5Y	378.7 ± 47.3 **
N-type	331.3 ± 47.3 **
S-type	568.0 ± 284.0 **

4.6.2. Effects of manganese on U373-MG cells

The U373-MG cells were more sensitive to manganese than the SH-SY5Y cells and especially RA was enhancing the toxicity of manganese (Figure 20). Both RA and manganese induced apoptosis in these cells and their joint effect seemed to be cumulative.

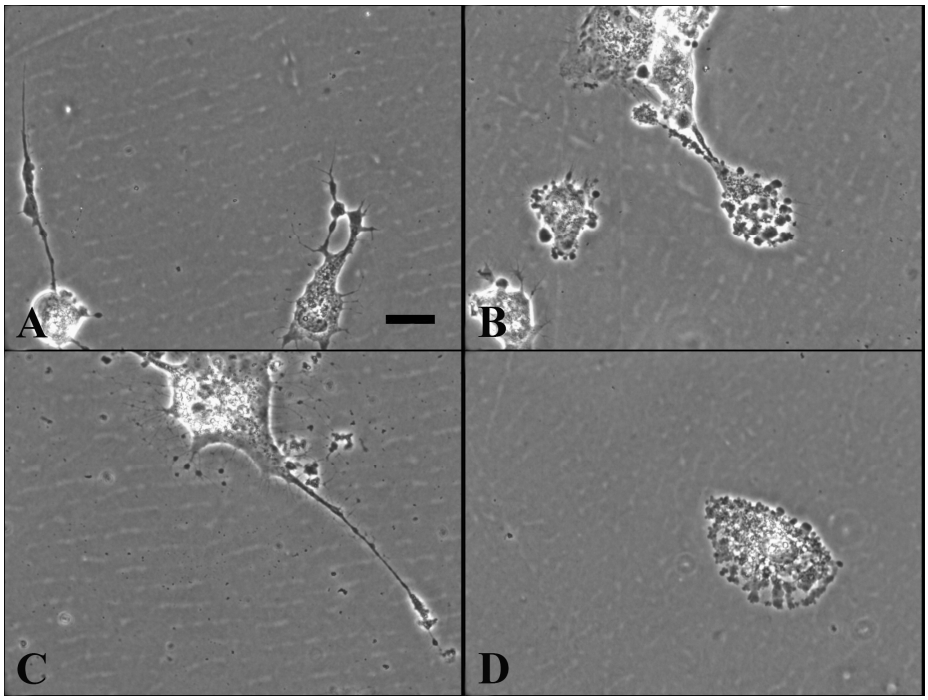

Figure 20. Mangnanese treatments in the U373-MG cells, where A is manganese, B is manganese with RA, C is manganese with cholesterol and D is manganese with RA + chol treated cells. The scalebar is 20 µm.

As can be seen from Figure 21, in the GFAP and vimentin labeled fluorescence images the cells treated with cholesterol appeared to be the most “normal” looking, though GFAP labeling was strong in all manganese treated cell cultures with or without RA and cholesterol.

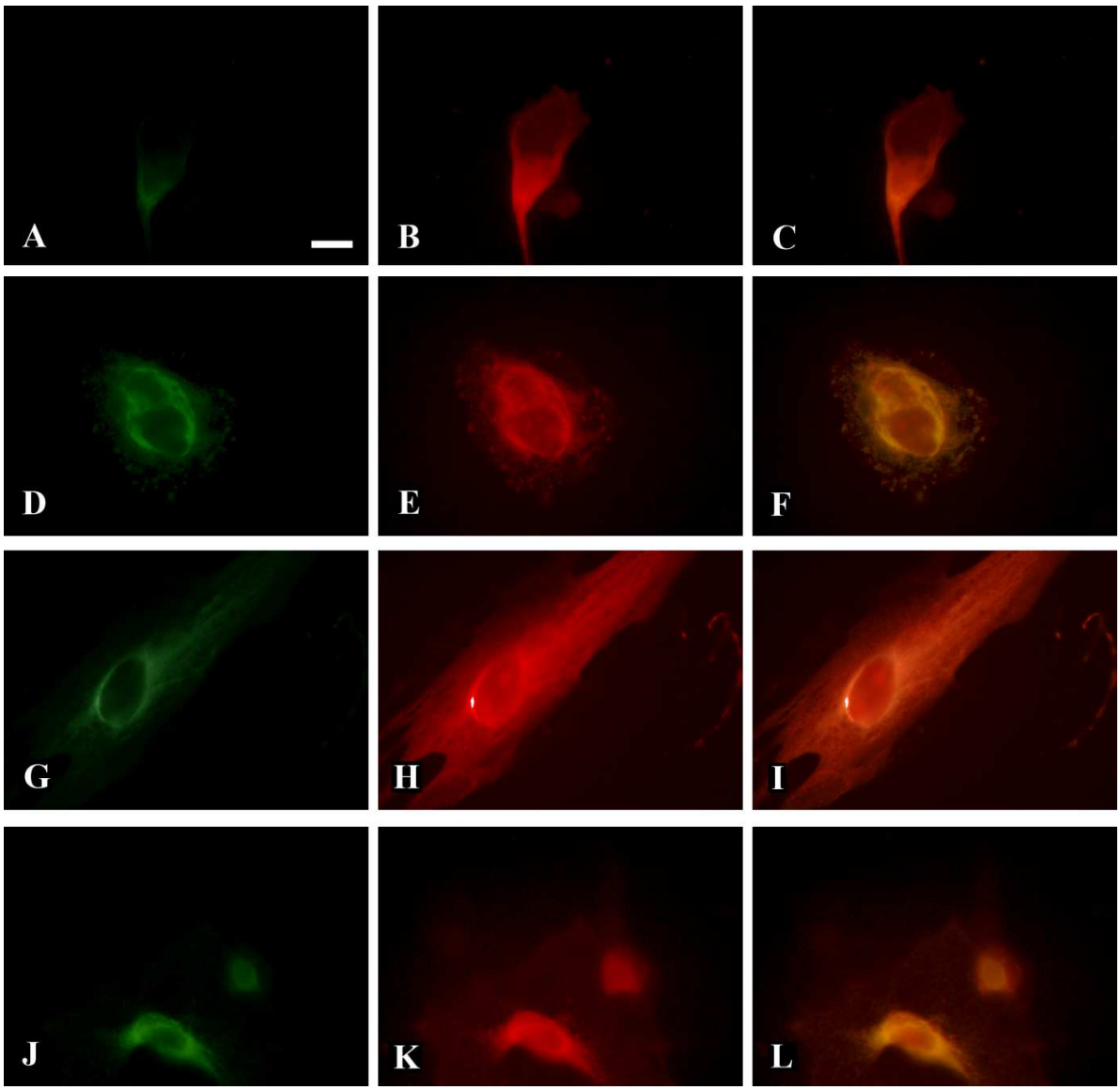

Figure 21. Fluorescence images of the human astrocytoma U373-MG cells, where A, D, G and J is GFAP, B, E, H and K is vimentin and C, F, I and L are merged images. Manganese treated cells are in A-C, manganese with RA treated cells are in D-F, manganese with cholesterol treated cells are in G-I and manganese with RA + chol treated cells are in J-L. The scalebar is 20 µm.

When cholesterol was added, more cells seemed to survive (Table V), though no statistically significant differences were seen (P = 0.062) between experiments with manganese with or without cholesterol. There was a statistically significant difference (P = 0.026) in the cell proliferation between manganese with cholesterol and manganese with RA + chol treatments, where there were less cells in the RA + chol treated cell cultures. In addition to manganese RA further reduced cell proliferation and cholesterol helped cells to survive from manganese-induced apoptosis.

Table V. Means and standard errors of the human astrocytoma cells in manganese, manganese with RA, manganese with cholesterol and manganese with RA + chol treatments. ** marks significant differences between manganese with chol and manganese with RA + chol treatment. N = 3 in each treatment.

treatment	Mean ± SE
Mn	601.3 ± 55.7
Mn + RA	710.0 ± 164.0
Mn + chol	994.0 ± 142.0 **
Mn + RA + chol	426.0 ± 82.0 **

5. Discussion

The main aim of this study was to develop an advanced method for separating the N- and S-type cells from the parental SH-SY5Y cell line. The distinct responses with RA, cholesterol and manganese treatments were observed with light and fluorescence microscopy, and the CNS tumor cell line U373-MG was used as a reference cell line with all introduced treatments.

5.1. Advanced method for SH-SY5Y cell phenotype separation

The method for phenotype separation earlier used by Biagiotti et al. (2006) was used as the initial methodology for this work. With this mechanical method the morphological changes of the cells eventually showed separation into the N- and S-type cells, but according to Biagiotti et al. (2006) it took 140 days to complete. To avoid the long culturing time, a faster method was developed in this study. The use of trypsin instead of PBS was cleaner and faster for the phenotype separation and with the new method separation of the two phenotypes could be clearly seen already after two weeks in culture. By this method the cells differentiate into N- or S-phenotype in a culture, where there are still interactions between different cells. Different cell phenotype separation methods usually include a limiting dilution of the cell suspension and then choosing one cell or one colony of cells for subcultures. However, these methods produce cells which are isolated clones, and do not resemble the “normal” transdifferentiating cells that appear in human neuroblastoma cell cultures. In the method developed in this study there are no isolations of single cells after the first separation, but removal of the “wrong” phenotype from the culture, allowing the “right” ones to remain. Also, the S-type cells have been hard to maintain in continuous cultures for longer times (Biedler et al., 1973; DeClerck et al., 1987; Rettig et al., 1987; Ciccarone et al., 1989; Marzi et al., 2007), so a method for a quick cell phenotype separation is needed to re-establish the wanted cell type *in vitro*.

The neuroblastoma SH-SY5Y cell line used in this study was the third N-type clone from the parental cell line SK-N-SH (Biedler et al., 1978), which still does not exclude the S-type cells (also observed by Encinas et al., 2000), and thus caution should be used when different cell lines are named to be of a certain phenotype. Initially the S-type cells resembled

more of the parental SH-SY5Y cells (Figure 5 A-C), but as the S-type cells were cultured for longer times, the flat cell morphology became the main phenotype and they seemed to resemble morphologically more of the astrocytoma U373-MG cells. The N-type cells resembled the parental SH-SY5Y cells, but in general were smaller and had more processes. During this study both the N- and S-type cells showed distinctly different phenotype around 8 DIV and this might indicate that the cells differentiate to their selected phenotype around this time. The developed new separation method produced morphologically differentiated cell type cultures in 14 days, compared to the 120 days introduced by Biagiotti et al. (2006).

As the human neuroblastoma cells are highly dependent on oxygen and metabolites *in vitro* (Hoehner and Prabhakaran, 2003), the occasional problems in cell culture may have been caused by low amount of medium used in this study. This may partly explain the low proliferation observed in the cell cultures. The passages 25-31 of the SH-SY5Y cells used for this study appeared to be generally slow in proliferation (personal communication, Sarkanen, University of Tampere, Tampere, Finland and Silvennoinen, University of Jyväskylä, Jyväskylä, Finland), and thus larger amount of cells in plating would have given better chance for cell growth.

5.2. Retinoic acid toxicity on SH-SY5Y cells

To evaluate how the differentiation induced by RA and supported by cholesterol affects the morphology of the different SH-SY5Y cell types separated in this study, the cells were treated with RA and cholesterol for up to 10 DIV. The concentration of retinoic acid used in this study has been shown not to be cytotoxic (Ross et al., 1995), though slight induction of apoptosis, especially in the S-type cells, has been noted (Ponzoni et al., 1995; Mäntymaa et al., 2000; Voigt and Zintl, 2003). However, in this study RA seemed to cause cell death, especially in the N-type cell cultures.

The RA treated cells generally showed less proliferation when compared to the non-treated cells. The significant differences seen only after 10 days in culture is in agreement with earlier results, where it has been shown how SH-SY5Y cell differentiation requires at least 5-7 days of RA treatment (Joshi et al., 2006; Sarkanen et al., 2007). Cultures treated with RA +

chol showed cell proliferation until 7 DIV. After this the number of cells decreased to same levels seen with RA alone treatments in all types of SH-SY5Y cultures, as was also observed by Sarkanen et al. (2007). However, the decrease in the cell proliferation in RA + chol treatments is not as radical as seen in treatments with RA alone, because cholesterol is beneficial for stabilizing the cells and when added to the cells together with RA, cells survive better from the RA-induced apoptosis.

5.3. S-type cell resistance to apoptosis

Marzi et al. (2007) observed that the S-type cells were more resistant to hypoxia and were not vulnerable to apoptosis, when compared to the N-type cells, and that over time the S-type cells outnumbered the N-type cells in these hypoxic conditions. Similar behavior was detected also in this study, as there were still S-type cells left in the N-type cell cultures, which were otherwise damaged by manganese and RA treatments. In this study the S-type cells proved to be most resistant to the apoptotic effects of manganese, which may indicate the more differentiated status of the S-type cells. Also, RA did not differentiate the S-type cells to the N-type cells, which corresponds to the observations by Messi et al. (2008).

Recently, a study showed how human neuroblastoma cells, when treated with RA and methyl mercury (MeHg) were more sensitive to the cytotoxic effects of MeHg (Kim et al., 2007). In addition, it was shown, that those cells, which were differentiated with RA together with MeHg addition, were more sensitive than cells already differentiated before adding the MeHg. Also, cells treated with RA have been reported to have elevated levels of MnSOD (manganese Super-Oxide Dismutase, located inside the mitochondria, Mäntymaa et al., 2000) which suggests, that RA could have similar antioxidant properties than manganese. Also, the mitochondria of the N-type cells are generally more complex structurally than the S-type cells mitochondria (Tumilowicz et al., 1970), which may be one reason for the vulnerability of the N-type cells to the manganese-induced apoptosis.

5.4. Cholesterol and cell survival

Human U373-MG astrocytoma cell line was used as a means to compare the effects of RA, cholesterol and manganese seen in the SH-SY5Y cells. Cells were grown similarly to the SH-SY5Y cells for up to 10 DIV, and treated with same amounts of RA, cholesterol and manganese. U373-MG cells were not growing steadily and never reached confluency with these plating densities and in these culture conditions. The surviving and proliferating U373-MG cells were large and granular. It is known, that the human astrocytoma cells exhibit different phenotypes, but these have not been studied in any detail (for a review see Miller and Perry, 2007). However, the U373-MG cells seen in the present study are suggested to be the occasionally observed giant type cells.

All fluorescence labels used (Hoechst 33258, vimentin and GFAP) stained U373-MG cells better than SH-SY5Y cells. It has been shown that GFAP labels the fibrillary acidic proteins in the human astrocytoma cells more intensively when the cells are in apoptosis or differentiated (Toimela and Tähti, 1995; Holden and Coleman, 2007), and this was observed also in this study. It has previously been shown, that the human astrocytoma cells are more resistant to cell death than human neuroblastoma cells (Higashi et al., 2004; Malthankar et al., 2004; Dukhande et al., 2006), but in this study the giant type U373-MG cells were extremely vulnerable to manganese. However, cholesterol had the same protecting effect on U373-MG cells as was seen with the SH-SY5Y cells. Cholesterol also induced the formation of thick and long processes in the U373-MG cells, the reason for which is unknown and needs further studies. Further studies, especially on the different responses of the human astrocytoma cell types with treatments with RA and cholesterol, as well as on the possible differential resistance to apoptosis, are also needed.

It has been reported, that patients treated with RA together with steroids have less cytotoxic symptoms from RA, and have elevated levels of cholesterol. It has also been suggested that cholesterol might have a protective effect to neuroblastoma cell toxicity (Yung et al., 1996). These strengthen the suggestion, that cholesterol could be used together with RA treatments, and combined administrations of these substances could provide more stable and efficient results in cancer therapies, where cholesterol might help to avoid inflammation and

reduce side effects. For the future clinical therapies the conversion of the malignant I- and N-type cells into the non-malignant S-type cells is one possibility. It has been suggested elsewhere, that the transdifferentiation between the S- and N-type cells could represent a model of tumor regrowth and regression (Chen et al., 2000). Also, Bian et al. (2002) suggest, that the S-type cells could determine the disease outcome. The results of the S-type cell resistance to different treatments suggest that it is not sufficient to only change the cells into one non-malignant phenotype, but to also remove those non-malignant S-type cells. Furthermore, induced apoptosis, especially to differentiated cells, could provide a therapy method when fighting against devastating CNS cancers.

5.5. Conclusions

A new method for separating the N- and S-phenotypes from the parental SH-SY5Y cell culture was here introduced, and the different responses of the N- and S-type cells to RA, cholesterol and manganese treatments were studied. In the SH-SY5Y cell cultures with different phenotypes, the treatments are designed to explain the differential behavior of the whole cancer with multiple cell types. The method developed in this study might offer an opportunity to study the cell phenotypes in a more natural environment, as the phenotypes are not isolates, but have had the opportunity to cell-cell interactions before the phenotype separation.

The N-type cells were found to be more vulnerable to the treatments with RA and manganese, when compared to the S-type cells, which would suggest that the N-type cells are more differentiated than the S-type cells. However, in contrast, the N-type cells are seen to dominate over the S-type cells *in vitro*, which suggest that the S-type cells are more differentiated and not dividing as actively as N-type cells. Also, the differentiating RA treatment does not seem to cause morphological changes in the S-type cells. The ability of the S-type cells to resist different treatments may depend on their ability to transdifferentiate back to the potential stem cell (the I-type cell). If the N-type cells have lost the ability to do so, it could be said, that the N-type cells are the most differentiated cells also *in vivo*. As the differentiation status of the N- and S-type cells is controversial, more research on this issue is needed.

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7. Appendices

7.1. Culture medium for SH-SY5Y cells

87 ml MEM (GIBCO Invitrogen, 21090, Carlsbad, CA, USA)

89 ml F-12K (GIBCO, 21127)

1 ml L-glutamine (1 mM, Immuno Dianostics, Wobum, MA, USA)

1 ml non-essential amino acids (1 %, PAA laboratories, Pasching, Austria)

20 ml FBS (10 %, PAA-laboratories)

3 ml antibiotics (1 %, streptomycin and ampicillin, PAA-laboratories)

7.2. Culture medium for U373-MG cells

170 ml MEM (GIBCO, 21090)

2 ml Na-pyruvate (1 %, GIBCO)

2 ml L-glutamine (1 mM, Immuno Diagnostics)

2 ml non-essential amino acids (1 %, PAA-laboratories)

20 ml FBS (10 %, PAA-laboratories)

2 ml antibiotics (1 %, streptomycin and ampicillin, PAA-laboratories)