Folia Biologica et Oecologica 12: 1–10 (2016)
Acta Universitatis Lodziensis

DNA methylation: gene expression regulation

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ABSTRACT

Epigenetic modifications are responsible for the modulation of gene expression without affecting the nucleotide sequence. The observed changes in transcriptional activity of genes in tumor tissue compared to normal tissue, are often the result of DNA methylation within the promoter sequences of these genes. This modification by attaching methyl groups to cytosines within CpG islands results in silencing of transcriptional activity of the gene, which in the case of tumor suppressor genes is manifested by abnormal cell cycle, proliferation and excessive destabilization of the repair processes. Further studies of epigenetic modifications will allow a better understanding of mechanisms of their action, including the interdependence between DNA methylation and activity of proteins crucial to the structure of chromatin and gene activity. Wider knowledge of epigenetic mechanisms involved in the process of malignant transformation and pharmacological regulation of the degree of DNA methylation provides an opportunity to improve the therapeutic actions in the fight against cancer.

KEY WORDS: transcriptional activity, epigenetics, carcinogenesis

Introduction

The human genome is composed of approximately three billion base pairs and contains large amounts of genetic information. Although different types of cells share the same DNA, they display different phenotypes. It indicates that regulated access to the genetic information plays an important role in understanding cell identity and, thus, human development (Sharma *et al.* 2010, Jurkowski *et al.* 2015). The term "epigenetics" was coined by Conrad Waddington and defined as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into

DOI: 10.1515/fobio-2016-0001

being" (Goldberg *et al.* 2007, Kunwor *et al.* 2015). Initially, this definition referred to epigenetics in context of embryonic development, however it has evolved over time and nowadays, epigenetics is described as "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence" (Sharma *et al.* 2010, Brait & Sidransky 2011). Most of these changes occur during differentiation and are maintained through multiple cell divisions, allowing cells to develop distinct identities despite having the same genetic information. Epigenetic modifications such as cytosine methylation, histone post-translational modifications as well as the nucleosome positioning along the DNA, mediate heritability of gene expression patterns (Goldberg *et al.* 2007, Carone *et al.* 2010, Greer *et al.* 2011). The set of these modifications, known as the epigenome, regulates the accessibility of the genetic information to the cellular machinery, providing a mechanism for cell diversity (Lee & Lee 2012). Failure to properly maintain epigenetic marks can result in disruption of different signaling pathways by their inappropriate activation or inhibition, and therefore, lead to disease such as cancer. Recent studies show that both genetic and epigenetic alterations are equally important and can contribute to all stages of human cancer development (Kresse *et al.* 2012, You & Jones 2012, Marquardt *et al.* 2013). In contrast to genetic mutations, epigenetic modifications are reversible, which makes them an attractive and promising target for cancer therapy (Esteller 2008, Khan *et al.* 2008, Sadikovic *et al.* 2008, Riggins 2014, Yang *et al.* 2014, Kunwor *et al.* 2015, Nakamura *et al.* 2015).

Methylation patterns in normal cells

Chromatin is composed of repeated structural units, known as nucleosomes, which consist of approximately 146 base pairs of DNA wrapped around a histone protein octamer made up of two copies of each of the four histone proteins such as H2A, H2B, H3 and H4 (Flis *et al.* 2007, Sharma *et al.* 2010, Lee & Lee 2012). DNA methylation, covalent and non-covalent histone modifications, non-coding RNAs including miRNAs are epigenetic modifications associated with alteration of the dynamics of chromatin structure, its accessibility and compactness. The distinct patterns of these modifications regulate the functioning of the genome and the way it manifests itself in different types of cells, stages of development and various diseases, including cancer, and thus protect the identity of the cell (Sharma *et al.* 2010).

DNA methylation is a reversible addition of a methyl group (-CH3) to either adenine or cytosine bases. In mammalian cells, methylation occurs at the fifth carbon of the cytosine pyrimidine ring within CpG dinucleotides that can be concentrated in short CpG-rich DNA regions known as CpG islands or regions of large repetitive sequences, such as retrotransposon elements and centromeres (Saxonov *et al.* 2006, Flis *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010). CpG islands are frequently located at the 5' regulatory regions of a gene and are associated with approximately 60–70% of human gene promoters. Methylation of the CpG island promoter, catalyzed by DNA methyltransferases (DNMTs) that use S-adenosyl-L-methionine as the donor of methyl groups, prevents binding of transcription factors which results in gene silencing (Saxonov *et al.* 2006, Łukasik *et al.* 2009, Guz *et al.* 2010). DNMT1, often referred to as the "maintenance" methyltransferase, is one of the three active DNA methyltransferases identified in mammals. It recognizes and binds to

hemimethylated CpG sites generated during DNA replication in which the parental strand remains methylated, unlike the newly synthesized one. In order to maintain existing CpG methylation patterns, DNMT1 attaches a methyl group to the cytosines on the daughter strand (Hirasawa *et al.* 2008). Two other methyltransferases, DNMT3A and DNMT3B, target previously unmethylated cytosines and establish DNA methylation patterns early in development, and therefore are called *de novo* methyltransferases (Flis *et al.* 2007, Heinz *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010, Ficz & Gribben 2014, Kunwor *et al.* 2015).

The pattern of DNA methylation is not only a consequence of attachment of methyl groups to cytosine but also DNA demethylation (Guz et al. 2010, Tan *et al.* 2012, Hill *et al.* 2014). Demethylation is a reaction of removal of the methyl group and can be considered as DNA replication-dependent and independent (Guz *et al.* 2010, Hill *et al.* 2014). This process requires several steps and the first one is oxidation of 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) with the participation of Tet proteins. It is assumed that the more diverse and stable the cell is, the less 5hmC can be expected. Hydroxylation of 5mC occurs most actively in the zygote and embryo stage, when parental methylation pattern is erased by Tet3 protein. Tet1 Tet2 proteins are active during embryogenesis, making it possible to maintain an adequate level of housekeeping gene expression and sufficient number of stem cells, inhibiting their differentiation (Tahiliani *et al.* 2009, Globisch *et al.* 2010, Tan *et al.* 2012, Hill *et al.* 2014). During the development of the embryo, in which cells divide intensively, 5-hydroxymethylcytosine is transcribed as unmodified cytosine, and therefore is not recognized by DNMT1. This process is called passive DNA demethylation, however can be also described as DNA replication-dependent, because it occurs when DNMT1 does not methylate newly synthesized DNA strand. In consequence, the second round of replication, which is not accompanied by maintenance methylation, results in a completely unmethylated DNA (Ficz & Gribben 2014, Arand *et al.* 2015). Active DNA demethylation plays an important role in cells that divide less often and can take place in several ways. One of them is further oxidation using Tet proteins, first to the 5-formylcytosine (5fC) and next to 5-carboxylcytosine (5caC), which must be subjected to decarboxylation. There is also the possibility of 5hmC glycosylation or deamination to 5-hydroxymethyluracil. In both cases, the modified nucleotide is considered to be invalid by the base excision repair system (BER) and replaced by cytosine. This is the way of CpG islands demethylation, usually located near the transcription initiation site, to which Tet1-3 proteins preferentially bind, preventing their secondary methylation (Wu & Zhang 2011, Tan *et al.* 2012, Hill *et al.* 2014).

In normal cells (Fig. 1), methylation usually occurs in repetitive regions associated with chromosomal stability, non-coding regions as well as in gene bodies. Although, the majority of CpG islands located in the promoter regions of genes are protected from this epigenetic mechanism and remain unmodified during the development and in differentiated tissues, some of them become methylated. The most classic examples of CpG island methylation during the development, resulting in long-term transcriptional silencing, are X-chromosome inactivation and gene imprinting (Flis *et al.* 2007, Kiefer 2007, Esteller 2008, Illingworth *et al.* 2008, Łukasik *et al.* 2009, Guz *et al.* 2010).

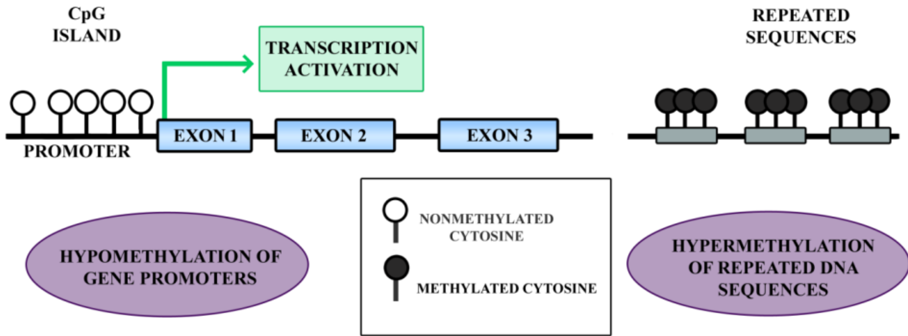

Figure 1. DNA methylation in normal cells.

DNA methylation in cancer cells

Hypermethylation of CpG islands and global hypomethylation are characteristic of cancer cells (Fig. 2). The low level of methylation in the rest of the genome can induce the activation of oncogenes located nearby and too frequent methylation within CpG islands - silencing of tumor suppressor genes (Flis *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010, Hansen *et al.* 2011, Yang *et al.* 2014, Kunwor *et al.* 2015).

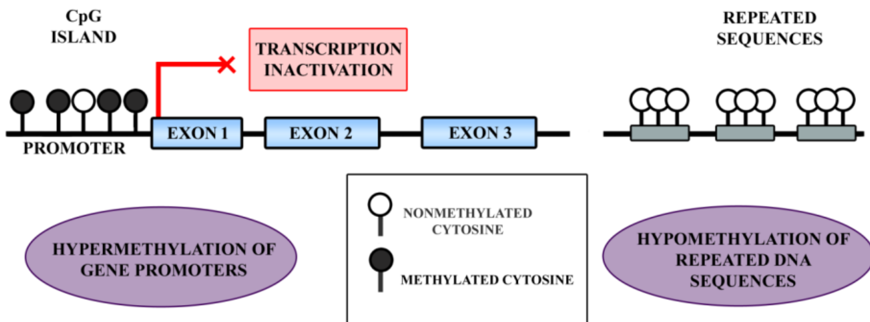

Figure 2. DNA methylation in cancer cells.

In colorectal cancer, the 10-30% reduction was observed in the overall methylation as well as significant reduction in the amount of 5-methylcytosine in premalignant stages of the adenoma (Wilson *et al.* 2007, Ehrlich 2009, King *et al.* 2014). Hypomethylation of over 50% was noted in the tumors of the chest (Wilson *et al.* 2007, Rauch *et al.* 2008). Hypomethylation in tumors of blood occurs in chronic lymphocytic leukemia

(CLL), whereas in the chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) and multiple myeloma there is only a small change in the pattern of DNA methylation (Stach *et al.* 2003, Lyko *et al.* 2004, Wilson *et al.* 2007). The global demethylation occurs in the early stages of tumors of the chest, colon and in chronic lymphocytic leukemia. In addition, in colorectal cancer hypomethylation is present in normal tissues adjacent to the tumor. In other tumors, eg. hepatocellular carcinoma hypomethylation increases with advancing stage and histological tumor stage (Lin *et al.* 2001, Wilson *et al.* 2007). Hypomethylation of specific genes was observed in the tumors of colon, pancreas, chest, stomach, prostate and in leukemia (Sadikovic *et al.* 2008). Usually, these genes regulate growth, encode enzymes important for the organism's development, tissue-specific genes and oncogenes (Flis *et al.* 2007, Guz *et al.* 2010, Kunwor *et al.* 2015).

The most common regions of hypermethylation in different kinds of tumors are chromosome 3p, 11p and 17p (Rush *et al.* 2001, Choi *et al.* 2007, Sulewska *et al.* 2007, Stöcklein *et al.* 2008). This phenomenon occurs within CpG islands which are normally unmethylated in the genome. The most important consequence of this event is silencing the function of tumor suppressor genes, for example promoter hypermethylation of p16 gene (INK4A), which occurs in many tumors. p16 is an inhibitor of cyclin-dependent kinase, which negatively regulates cell cycle progression from G1 to S phase (Flis *et al.* 2007, Li *et al.* 2011). Abnormal expression leads to disruption of the cell cycle and the loss of control, which stimulates proliferation and affect tumor progression. This phenomenon was noted in bladder, nose, throat, pancreas, colon, lung cancers as well as in melanomas, leukemias and glioblastomas. In the carcinogenesis of esophageal adenocarcinoma promoter methylation of the p16 gene can occur already in the metaplasia (Auerkari 2006, Li *et al.* 2011). In addition, the repression of transcription of another gene, MLH1 encoding DNA mismatch repair protein, increases the frequency of mutations and, therefore, the abnormal expression of other genes (Tsai & Baylin 2011). Hypermethylation profile of 15 cancers such as colon, stomach, pancreas, liver, kidney, lung, head, neck, breast, ovary, bladder, endometrium, brain, lymphoma and leukemia was examined. Analysis consisted of 3 groups of genes: tumor suppressor genes: p16, p15, p14 (cyclin-dependent kinase inhibitors), p73 (p53-related protein), APC (adenomatous polyposis coli protein) and BRCA1 (breast cancer type 1 susceptibility protein); genes responsible for DNA repair or metabolism of xenobiotics: hMLH1, GSTP1 (glutathione S-transferase pi-1), MGMT (O^6-methylguanine DNA methyltransferase); genes involved in invasion and metastasis: CDH1 (cadherin-1), TIMP3 (metalloproteinase inhibitor 3), DAPK (death-associated protein kinase). Methylation in at least one gene was present in every type of tumor. Methylation profiles were dependent on both the gene and the tumor. Some genes, for example p16, MGMT, DAPK were methylated in various types of cancer (colon, lung, head, neck, ovary, bladder, lymphoma and leukemia) (Esteller *et al.* 2001, Flis *et al.* 2007). Hypermethylation of p14, APC, p16, MGMT, hMLH1 occurred in gastrointestinal tumors (colon, stomach) and GSTP1 in steroid tumors (breast, liver, prostate). Another study confirmed these reports. Methylation depends on the type of cancer for the following genes: BRCA1 - breast and ovarian cancer, hMLH1 - rectal, endometrial, gastric

cancer, p73 and p15 in leukemia (Flis *et al.* 2007, Esteller 2008).

Methods of detection and potential therapies

Detection methods must have a high sensitivity due to the material from which the DNA is isolated, and the specificity to distinguish methylation of tumor cells from methylation present in normal cells. None of the methods is universal and during the selection attention should be paid to the type, quantity and quality of the biological material. The correct choice of method should minimize the risk of contamination of the sample and ensure reproducibility of results (Łukasik *et al.* 2009). The most commonly used methods are: REP (restriction enzyme PCR), MS-PCR (methylation specyfic PCR), BSSCP (bisulfite single-strand conformation polymorphism), BGS (bisulfite genomic sequencing) (Majchrzak & Baer-Dubowska 2009, Łukasik *et al.* 2009). There are also other methods: MS-nested PCR, QAMA (quantitative analysis of methylated alleles), Heavy Methyl. The main objective of the analysis is the differentiation of methylated and unmethylated sequences. This can be achieved either by using methylation sensitive restriction enzyme or chemical modification of DNA by sodium bisulphite. Sodium bisulfite deaminates cytosine to uracil, also m5C can undergo this reaction, however, very slow formation of the intermediate product significantly limits the speed of the process. Defined DNA fragments are then subjected to allele-specific PCR (MS-PCR), SSCP (BSSCP) or sequencing (BGS) (Łukasik *et al.* 2009).

DNA methylation pattern of adults is tissue specific and relatively stable. It is known that it can be changed in the early stages of embryonic development, during cell differentiation. Significant changes in the profile of DNA methylation are commonly detected in cancer cells (Ogoshi *et al.* 2011, You & Jones 2012). In many tumors it has been shown that inactivation of tumor suppressor genes is accompanied by hypermethylation of the promoter regions. Hypermethylation within CpG islands which are normally unmethylated in the genome, is a factor that inhibits transcription and expression of genes (Deaton & Bird 2011). Considering that tumor suppressor genes are involved in cell differentiation and regulation of the cell cycle, apoptosis and repair of DNA, the consequences of hypermethylation of the promoter sequences resulting in silencing genes are evident. Therefore, compounds which inhibit DNA methylation can play a role in tumor therapy (Guz *et al.* 2010, Kunwor *et al.* 2015).

The best known inhibitors of DNA methyltransferases (DNMTs) are cytidine analogues modified in the 5 position of the pyrimidine: 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine) (Flis *et al.* 2007, Guz *et al.* 2010, Kunwor *et al.* 2015). The mechanism of the pharmacological action of these compounds is their conversion in cells to deoxynucleotide triphosphates and then incorporation into DNA in a place of cytosines during replication (Brait & Sidransky 2011). This modification is recognized by DNMT to which it binds covalently, blocking its activity. Formation of the enzyme-DNA adducts reduces the number of active DNMT molecules in the nucleus, which in subsequent rounds of replication result in passive methylation of DNA, and therefore in the reactivation of epigenetically silenced genes. Covalent binding of DNA methyltransferases may be responsible for the cytotoxicity of the DNMT inhibitors, especially in high doses. Low stability in aqueous solutions and high toxicity of azanucleosides greatly limits their therapeutic potential

(Flis *et al.* 2007, Guz *et al.* 2010). Another cytidine analogue lacking the amino group at C4 of the pyrimidine ring is Zebularine, which has a similar mechanism of action to azanucleosides. Zebularine is a compound less toxic than 5-azacytidine and decitabine, and more stable in aqueous solutions, however, its bioavailability after oral administration is rather low (Cheng *et al.* 2003, Guz *et al.* 2010, Sharma *et al.* 2010, Kunwor *et al.* 2015). Another group of compounds that inhibits DNMTs activity are small molecule inhibitors, including hydralazine (an antihypertensive action), procaine (local anesthetic) or procainamide (antiarrhythmic drug). Procaine and procainamide are derivatives of 4-aminobenzoic acid and are capable of annealing to a sequence rich in CpG, causing the masking target sequences for methyltransferase and thus block the binding of the enzyme with DNA (Guz *et al.* 2010, Kunwor *et al.* 2015). The group of inhibitors, that are not nucleoside analogues, includes the compounds directly blocking the activity of DNA methyltransferase, such as epigallocatechin gallate (EGCG), which is considered to be the most active of green tea polyphenols and L-tryptophan derivative (RG108). The mechanism of action of these compounds consists in blocking the active center of the enzyme. RG108 because of its good fit to the active center of DNMT1 and low toxicity was an attractive candidate for further research on the use of anticancer therapy, however it has been noted that RG108 is genotoxic (Kunwor *et al.* 2015). An alternative mechanism of DNMT inhibition could be the use of antisense oligonucleotides directed against the DNMT mRNA. Hybridization of an antisense oligonucleotide with the complementary mRNA may block the translation, thus reduce the level of DNA methyltransferases (Flis *et al.* 2007, Guz *et al.* 2010).

Conclusions

DNA methylation plays an important role in the complex and multistep regulation of expression of the genes, whose promoter regions are rich in CpG sequences. The above data indicate that the methylation and gene expression are processes related to each other by several factors, such as the activity of DNA methyltransferases factors transcriptionally, proteins involved in demethylation, protein binding methylated DNA. Further studies of epigenetic processes will allow a better understanding of mechanisms of their action, including the interdependence between DNA methylation and activity of proteins crucial to the structure of chromatin and gene activity. Wider knowledge of epigenetic mechanisms involved in the process of malignant transformation and pharmacological regulation of the degree of DNA methylation provides an opportunity to improve the therapeutic actions in the fight against cancer.

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Streszczenie

Modyfikacje epigenetyczne odpowiedzialne są za modulację ekspresji genów bez ingerencji w sekwencję nukleotydową. Obserwowane zmiany aktywności transkrypcyjnej genów w tkankach nowotworowych w porównaniu do tkanki prawidłowej, bardzo często są wynikiem metylacji DNA w obrębie sekwencji promotorowych tych genów. Modyfikacja ta poprzez przyłączenie grup metylowych do cytozyn wysp CpG skutkuje wyciszeniem aktywności transkrypcyjnej genu, co w przypadku genów supresorowych przejawia się zaburzeniami cyklu komórkowego, nadmierną proliferacją i destabilizacją procesów naprawczych. Dalsze badania nad modyfikacjami epigenetycznymi pozwolą na lepsze zrozumienie mechanizmów ich działania, w tym zależności pomiędzy metylacją DNA, a aktywnością białek decydujących o strukturze chromatyny i aktywności genów. Poszerzanie wiedzy na temat epigenetycznych mechanizmów biorących udział w procesie transformacji nowotworowej i farmakologicznej regulacji stopnia metylacji DNA może stanowić okazję do poprawy działań terapeutycznych w walce z nowotworem.

Folia Biologica et Oecologica 12: 11–25 (2016)
Acta Universitatis Lodziensis

Endocannabinoid system and anticancer properties of cannabinoids

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ABSTRACT

Cannabinoids impact human body by binding to cannabinoids receptors (CB1 and CB2). The two main phytocannabinoids are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC interacts with CB1 receptors occurring in central nervous system and is responsible for psychoactive properties of marijuana. CBD has low affinity to CB1 receptor, has no psychoactive characteristics and its medical applications can be wider. CB receptors are part of a complex machinery involved in regulation of many physiological processes – endocannabinoid system. Cannabinoids have found some applications in palliative medicine, but there are many reports concerning their anticancer affects. Agonists of CB1 receptors stimulate accumulation of ceramides in cancer cells, stress of endoplasmic reticulum (ER stress) and, in turn, apoptosis. Effects of cannabinoids showing low affinity to CB receptors is mediated probably by induction of reactive oxygen species production. Knowledge of antitumor activity of cannabinoids is still based only on preclinical studies and there is a necessity to conduct more experiments to assess the real potential of these compounds.

KEY WORDS: cannabinoids, cancer, tetrahydrocannabinol, THC, cannabidiol, CBD

Introduction

Cannabinoids are diverse lipophilic compounds which interact with cannabinoid receptors (CBRs) in mammal body. This group of chemicals can be divided into three main classes: phytocannabinoids, endocannabinoids and synthetic cannabinoids. Phytocannabinoids naturally occur in plants of *Cannabis* genus. More than 60 cannabinoids are identified in *Cannabis sativa*, of which the most abundant are Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC) and cannabigerol (CBG). THC is the main psychoactive component of marijuana – natural product obtained by

DOI: 10.1515/fobio-2016-0002

drying flowers and leaves of *C. sativa* and *C.indica*. THC strongly impacts central nervous system (CNS) by binding to CB1 receptors and exhibits euphoric, analgesic and antiemetic properties. Another important constituent of *Cannabis*, cannabidiol (CBD) has low affinity to CB receptors and show no psychoactive characteristics (Fig. 1). Its effects are mediated by other receptor types.

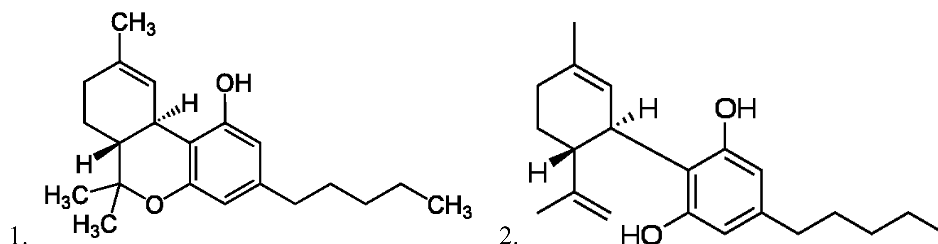

Figure 1. Examples of phytocannabinoids. 1 - Δ^9-tetrahydrocannabinol (THC); 2 - cannabidiol (CBD).

Second main group, endocannabinoids includes endogenous ligands of CB receptors which are part of endocannabinoid system. Endocannabinoid system present in mammal body is involved in modulation of many physiological processes, like inflammation, memory or pain modulation. The best characterized endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Fig. 2).

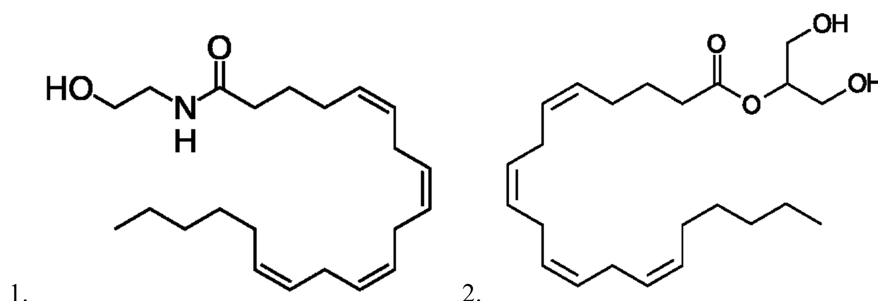

Figure 2. Examples of endocannabinoids. 1 - anandamide (AEA); 2- 2-arachidonoylglycerol (2-AG).

Third group is constituted by synthetic cannabinoids, compounds which mimic properties of natural cannabinoids.

Variety of physiological processes in which endocannabinoid system is engaged causes that affecting its activity by phytocannabinoids or synthetic ligands of CBRs is a promising therapeutic strategy in many diseases. Cannabinoids-based preparations have found some applications in palliative medicine. Nabiximols, oromucosal spray which contains THC and CBD in about 1:1 ratio is allowed in some countries for treatment of spasticity in multiple sclerosis. Dronabinol (synthetic THC in form of capsules) is allowed in USA and Germany for treatment nausea and vomiting associated with chemotherapy

and for anorexia in patients with AIDS. Nabilone (synthetic analogue of THC, capsules) can be used in USA, UK, Mexico and Austria also for nausea and vomiting associated with chemotherapy (Whiting *et al*. 2015).

Another important branch of cannabinoids research concerns their anticancer effects. First reports on the antiproliferative properties of THC comes from years 1975 and 1976. It has been shown that THC inhibits lung adenocarcinoma proliferation *in vitro* and tumor growth in murine model (Munson *et al*. 1975, White *et al*. 1976). Since that time there has been collected a lot of data referring to anticancer characteristics of cannabinoids, both *in vitro* and *in vivo* in cases of glioblastoma multiforme, breast, prostate, thyroid, colon, pancreas cancer or leukemia and lymphoma (Pisanti *et al*. 2009). It includes action of endocannabinoids (AEA, 2-AG), phytocannabinoids (THC, CBD) as well as synthetic cannabinoids (JWH-133, WIN 55,2121-2). Many studies have shown that cannabinoids can inhibit proliferation of cancer cells, induce apoptosis/autophagy, inhibit angiogenesis and formation of metastasis (Velasco *et al*. 2012).

Mechanism of cannabinoids anticancer action is complex and many of its parts are still waiting to be fully elucidated.

Methods

Review of the available literature was done. We used PubMed database. Besides the latest reports, we consider also some older papers concerning the first discoveries of anticancer properties of cannabinoids.

Endocannabinoid system

Cannabinoids affect cells mainly through two classical receptors belonging to the G Protein-Coupled Receptor (GPCR) superfamily: CB1 and CB2, which are part of the endocannabinoid system, involving cannabinoid receptors, theirs endogenous ligands (endocannabinoids) and enzymes engaged in synthesis, transport and degradation of cannabinoids (Hermanson & Marnett 2011). Activation of CB receptors leads to inhibition of adenylyl cyclase, which causes decrease in production of cyclic adenosine monophosphate (cAMP) and in turn activation of mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Bowles *et al*. 2012).

Endocannabinoids act as retrograde transmitters: they are synthesized by postsynaptic cells in answer to binding neurotransmitters and diffuse through the synaptic gap to the presynaptic membrane where bind to CB receptors, which in turn leads to decrease in neurotransmitters release. Of note, endogenous cannabinoids are not stored in vesicles like other neurotransmitters. They are derived from arachidonic acid from plasma membranes (Stella *et al*. 1997).

As mentioned earlier, the two main endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In predominant pathway of AEA biosynthesis, arachidonic acid (AA) is transferred from phosphatidylchilne (PC) to phosphatidyletanolamine (PE) by N-acyltransferase (NAT) enzyme, which leads to formation of arachidonoyl phosphatidylethanolamine (NAPE). Then, NAPE is hydrolyzed to AEA by NAPE-selective phospholipase D (NAPE-PLD) (Wang & Ueda 2009, Bisogno *et al*. 1999).

2-arachidonoylglycerol is generally formed by hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) by

phospholipase C-β (PLC-β). DAG is in turn hydrolyzed to 2-AG by diacylglycerol lipase (DAGL) (Hermanson & Marnett 2011, Murataeva *et al*. 2014).

Described endocannabinoids are degraded by hydrolysis to an arachidonic acid: AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) and 2-AG by monoacylglycerol lipase (MAGL) (Hermanson & Marnett 2011).

CB1 and CB2 receptors belong to the A class (rhodopsin-like receptors) of G Protein-Coupled Receptor (GPCR) superfamily. Their amino acid sequence similarity is 44% (Pertwee *et al*. 2010). CB receptors are phylogenetically the closest related to lysophospholipid receptors (S1P, S1P1, S1P2, S1P3, S1P4, S1P5, LPA1, LPA2, LPA3), melanocortin 3 receptors (MC1-MC5), adenosine receptors (A1, A2A, A2B, A3) and the orphan receptors GPR3, GPR6 i GPR12 (Elphick & Egertová 2001, Fredriksson *et al*. 2003, Elphick 2007).

It is assumed that these groups of receptors emerged as a result of multiple duplications of one member of GPCR superfamilly. Orthologous receptors were identified only in *chordata* phylum, therefore the duplication which led to a creation of CB receptors took place most likely in the common ancestor of *chordata* (Elphick 2002, Elphick 2007, Elphick *et al*. 2003, Elphick & Egertová 2005).

Mechanism of action of both cannabinoid receptors relies on activation of $G_{i/o}$ proteins causing adenylyl cyclase inhibition and on activation of MAPK pathway by $G_{\beta\gamma}$ complex. Furthermore, CB1 receptor inhibits voltage-dependent calcium channel (VDCC) (Pertwee *et al*. 2010, Hermanson & Marnett 2011).

CB1 receptor is expressed mainly pre-synaptical at central end peripheral neurons, especially in central nervous system regions engaged in control of motility, memory and learning, emotions, perception, endocrine functions and analgesic effects (Velasco *et al*. 2012, Pertwee *et al*. 2010). It is responsible mainly for inhibition of neurotransmitters release. CB2 receptor is present mainly in immune cells and largely in microglia cells. It mediates modulation of cells migration and cytokine release (Pertwee *et al*. 2010, Cabral *et al*. 2008). Cannabinoid receptors are immunosuppressive.

Expression of CB receptors has been shown in many types of cancer cells, however its level is not always correlated with expression level in their tissues of origin (Fernández-Ruiz *et al*. 2007, Velasco *et al*. 2012).

Other receptors

Besides CB, there are many other, non-classical receptors which can interact with cannabinoids. The most important groups are vanilloid transient receptor potential cation channels (TRPV) and some orphan G protein coupled receptors.

TRPV1 receptors belong to the transient receptor potential (TRP) family of ion channels. They are formed by six transmembrane domains, contain cytosolic C- and N-terminal domains and non selective, kation-permeable region between fifth and sixth domains (Owsianik *et al*. 2006). Members of TRP family are engaged in many stimuli transduction, like temperature, electric potential, light, mechanic stimuli, flavor and savor, they mediate the effects of xenobiotic substances and endogenous lipids (Venkatachalam & Montell 2007). It has been shown that their expression level is frequently elevated in pathologically changed tissues (Nilius *et al*. 2007).

TRPV1 channel was firstly identified as capsaicin receptor, which is responsible for chilli pepper flavor

(Caterina *et al*. 1997). It is activated by many harmful factors as high temperature or low pH and is responsible for nociception (Caterina *et al*. 2000, Davis *et al*. 2000) TRPV1 receptor is localized mainly in sensory neurons but is also present in many others cells like lymphocytes or fibroblasts (Starowicz *et al*. 2007).

Some endocannabinoids and phytocannabinoids (CBD, CBG) can bind to TRPV1 receptors with high affinity and act as their full agonists (Starowicz *et al*. 2007, Bisogno *et al*. 2001, Ligresti *et al.* 2006). TRPV1 activation by cannabinoids can lead to the increase in concentration of reactive oxygen species and calcium, as well as cytochrome C release from mitochondria, which eventually leads to apoptosis (Maccarrone *et al*. 2000). It has been shown that activation of TRPV1 receptor by anandamide can induce apoptosis in neuroma, lymphoma and cervix cancer cells (Maccarrone *et al*. 2000, Contassot *et al*. 2004). Cannabidiol can exert anti-imflammatory effect through TRPV1 activation which causes inhibition of cyclooxygenase 1 and 2 (COX-1/2) (Hegde *et al*. 2011, Ruhaak *et al*. 2011)

G-protein-coupled receptors 55 are presumably a new group of CB receptors, but there is still not enough data about theirs interactions with cannabinoids to classify they as CBR. They belong to the A class of GPCR superfamily and have low sequence similarity to CB1 (13,5%) and CB2 (14,4 %) (Pertwee *et al*. 2010).

Cannabidiol acts as an antagonist of GPCR55 and competes with its endogenous ligand – lysophosphatidylinositol (LPI). Agonists of GPCR55 were shown to promote development of cancer in several model, therefore CBD can inhibit proliferation of cancer cells by preventing activation of these receptors (Andradas *et al*. 2016, Piñeiro *et al*. 2011, Hu *et al*. 2011). LPI stimulates proliferation of cancer cells by initiation of ERK, Akt pathways and release of Ca^{2+} (Piñeiro *et al*. 2011). There are observations showing correlations between GPCR55 expression level and rate of cancer development (Andradas *et al*. 2011, Pérez-Gómez *et al*. 2012).

It has been shown that cannabinoid receptors are able to associate with other receptors of GPCR superfamily, like dopamine, opioid or orexin receptors, forming heteromeric complexes. These associations probably can influence agonist's effect through allosteric interactions (Pertwee *et al*. 2010). That phenomenon can be responsible for some of the cannabinoids' biological effects.

Endocannabinoids show also pro-nociceptive action being transformed into prostaglandins which interact with prostaglandin receptors (Davis 2014).

Anticancer properties of CB receptors agonists

There are number of ways in which cannabinoids can impact cancer cells and which at least partially underline their antiprolifertive and proapoptotic properties. Firstly, activation of either cannabinoid receptors CB1 and CB2 leads to an activation of ceramide synthase, the enzyme that catalyzes synthesis of lipid molecules ceramides. Increase in ceramide concentration may be induced also by activation of sphingomyelinase, enzyme which causes release of ceramide from membrane sphigolipids (Calvaruso *et al*. 2012). Ceramides induce upregulation of an extracellular regulated kinase (ERK) signalling pathway which in turns causes apoptotic cell death (Sarfaraz *et al*. 2006, Sarfaraz *et al*. 2008). This process has been observed in gliomas, mantle cell lymphomas, colon and pancreatic cancers (Gustafsson *et al*. 2006, Guzmán *et al*.

2006, Cianchi *et al*. 2008, Carracedo *et al*. 2006).

Ceramide production lads also to endoplasmic reticulum stress (ER stress) which is connected with p8 protein expression (nuclear protein 1, Nupr1, transcription regulator involved in cancer development regulation), which in turn leads to the activation of TRIB3, inhibition of pAkt/mTOR and induction of apoptosis and autophagy (Velasco *et al*. 2012, Salazar *et al*. 2009, Sui *et al*. 2013, Salazar *et al*. 2013). Accumulation of ceramide cauces also long-term activation of Raf1/ERK cascade and inhibition of JNK (Hermanson & Marnett 2011). In this pathway a crucial role is played by the mitogen activated protein kinases (MAPK), which are serine – threonine kinases. They take part in transduction of extracelluar stimuli inside the cell and mediate in many diverse cellular responses, like cell cycle arrest, apoptosis or cytokine production. Much data has been collected confirming activation of kinases connected with response for extracellular stimuli in cases of proliferation inhibition of cancer cells by cannabinoids (Galve-Roperh *et al*. 2000). Long term upregulation of MAPK leads to activation of cyclin kinase inhibitor (p27/KIP1) which regulates signaling molecules crucial in cell cycle regulation (cyclines, cdk) and thereby induces cell cycle arrest and apoptosis (Kogan 2005, Sarfaraz *et al*. 2006, Sarfaraz *et al*. 2008). On the other hand in the cases of certain prostate and ovary cancer cell lines, activation of MAPK pathway by GPCR55 receptor can sustain proliferation (Piñeiro *et al*. 2011).

Ceramides also mediate in activation of a p38 mitogen-activated protein kinase (p38MAPK) pathway, upregulation of which also can lead to apoptosis through cytochrom C release from mitochondria or activation of caspases (Ramer & Hinz 2008).

Activation of apoptosis requires also inhibition of survive factors effects. Important signaling factor which mediate in action of survival factors is PI3K/Akt/mTOR pathway. This pathway is involved in many key processes, like cell survival, growth, proliferation, angiogenesis or cell migration (Hers *et al*. 2011). Inhibition of Akt kinase leads to cell cycle arrest and subsequently to apoptosis. Decrease in Akt activity is involved in cancer cell response to cannabinoids. This process has been observed in gastric cancer cells: CB receptors activation has led to MAPK pathway activation, Akt inhibition and cell cycle arrest (Park *et al*. 2011).

Another important issue is also that anticancer activity of cannabinoids can be stopped by pharmacological locking of each CB receptor in some cancer (gliomas) when in other tumors (pancreatic, breast, liver) it has been observed that only CB2 agonists have capacity to prevent induction of apoptosis (Galve-Roperh *et al*. 2000, Caffarel *et al*. 2006, Vara *et al*. 2011, Carracedo *et al*. 2006). These reports suggest that cannabinoids activate partially different metabolic pathways in different cancer types.

Anticancer action of non-psychoactive cannabinoids

Not all cannabinoids affect cells through CB receptors. Some cannabinoids do not bind with them at all or have very low affinity. The most widely studied is cannabidiol (CBD). It has low affinity to CB receptors, moreover, acts as a CB1 receptor antagonist. Therefore it shows no psychoactive properties by itself and blocks the psychoactive effect of THC and other CB1 receptor agonists. This characteristic makes this compound having high pharmacological potential

and in future can become valuable supplement in anticancer treatment.

Many CBD-binding receptors have been discovered but probably most of them do not mediate in its anticancer properties: GPR55, GPR18, 5HT1A, TRPV1, TRPV2, TRPM8, TRPA1, PPARγ, VDAC1 channel or mitochondrial sodium-calcium exchanger (Rimmerman *et al*. 2013a, Fernández-Ruiz *et al*. 2013, De Petrocellis *et al*. 2011, O'Sullivan & Kendall 2010).

In contrast to THC molecular and cellular mechanism of action of CBD is still not fully elucidated. The most frequent proposed mechanism of CBD action *in vitro* is induction of reactive oxygen species (ROS) production (De Petrocellis *et al*. 2013, McAllister *et al*. 2010, Shrivastava *et al*. 2011, Ligresti *et al*. 2006). ROS are side products of oxygen metabolism and play important role in signaling and homeostasis. Their production is correlated with proliferation of healthy cells and takes part in activation of metabolic pathways connected with growth (Benhar *et al*. 2002). On the other hand, reactive oxygen species can induce programmed cell death. It has been shown that ROS stimulate many factors involved in activation of apoptosis, like MAP3K5, JNK, p38 and activation of p53 pathway (Laurent *et al*. 2005, Benhar *et al*. 2001). Type of effects induced by ROS probably depends on rate and way of their production and on activity of antioxidative enzymes (Laurent *et al*. 2005).

First reports of ROS mediation in cannabidiol action come from 2004 (Massi *et al*. 2004). It has been demonstrated that CBD inhibits viability of glioblastoma multiforme cells by induction of apoptosis and this effect was abolished in the presence of α-tocopherol (α-TOC, antioxidant). Increase of ROS production was correlated with decrease in concentration of intracellular glutathione, that acts as important antioxidant. CBD effect was also selective – decrease in viability of healthy cells was not observed. Many later studies have shown similar mechanism of CBD action in other cancer cell lines, like breast cancer, prostate adenocarcinoma or leukemia (Mckallip *et al*. 2006, Massi *et al*. 2006, Mc kallip *et al*. 2006). ROS mediation in cannabidiol effects was confirmed in many experiments with the use of antioxidants like α-TOC or acetylcysteine. At the same time most of reports suggest that CBD affects cells without interactions with classical CB receptors or TRPV1 receptor (McAllister *et al*. 2015).

The way of ROS induction by CBD is still insufficiently discovered, but it is frequently indicated that there is a correlation between ROS production and an increase in intracellular Ca^{2+} concentration leading to changes in mitochondrial membrane potential. This effect was observed in breast cancer cells, hippocampus cells, oligodendrocytes and microglia (Rimmerman *et al*. 2013b, Ryan *et al*. 2009, Ligresti *et al*. 2006, Mato *et al*. 2010). Studies have shown that increase in Ca^{2+} results from releasing it from intracellular supplies and that ROS production induced by CBD is inhibited by chelating factor BAPTA-AM, which confirms calcium mediation in described effects (Ligresti *et al*. 2006).

The phenomenon which occurs after ROS induction in metabolic cascade induced by CBD is endoplasmic reticulum stress (ER stress). It has been observed that high level of ROS induces ER-stress by elevation of activity of many mediators like p8, CHOP, TRB-3 or GRP-78, which in turn triggers the

intrinsic pathway of apoptosis (Malhotra & Kaufman 2007).

Endoplasmic reticulum stress is complex signaling pathway triggered in response to stimuli including oxidative damage, hypoglycemia, viral infections or exposition to anticancer drugs. This process leads to inhibition of protein load on endoplasmic reticulum as a result of temporal suppression of translation and concomitant elevation of protein folding related genes expression. If these changes fail to restore homeostasis in ER, cell runs apoptosis or autophagy (Schröder & Kaufman 2005, Verfaillie *et al*. 2010).

Autophagy is a process of enclosing parts of cytoplasm in membrane vesicles called autophagosomes. Autophagosomes undergo fusion with lysosomes which leads to degradation of their content by lysosomal enzymes. Autophagy can play different roles in different circumstances. It allows cell recycling of damaged organella or triggers cell survive pathways, but it can also coexist or substitute an apoptosis in process of cell death (Mizushima *et al*. 2008). There are many reports concerning induction of autophagy process by cannabinoids in various cancer models and they indicate that this process partially shares signaling pathways with apoptosis. Cannabinoids induced autophagy was observed in glioma, melanoma, breast cancer, pancreatic cancer and liver cancer cells (Calvaruso *et al*. 2012).

Discussion

Despite data collected in many pre-clinical trials which suggests that cannabinoids have certain medicinal and anticancer potential, and can be used as supplementary drug in many diseases, there were conducted only few clinical trials. One of the reasons is that in many countries law regulations are unfavorable in terms of medical applications of cannabis. United States agency Drug Enforcement Administration (DEA) which controls use of substances with addictive potency, has placed marijuana and canabinoids which are CB1 receptors agonists in the Schedule I in Controlled Substances Act, which means that these substances are illegal in the USA. Schedule I substances are characterized by high abuse potency, no medical applications and no sufficient safety level for medical use (Office of Diversion Control 2016). This group includes also drugs like heroin, MDMA (ecstasy) or LSD. This classification is one of the reasons for the difficulties in clinical trials of cannabinoids. Medical communities of the US recommend re-evaluation of cannabinoids and change in their classification in order to facilitate research on the medical use of cannabis and cannabinoids (Bowles *et al*. 2012). However, many states have attempted to legalize cannabis-based medicines and to date marijuana is allowed for medical applications in 24 states and the District of Columbia (Birdsall *et al*. 2016).

So far, no clinical trial concerning anticancer properties of cannabinoids was conducted (National Cancer Institute 2016). The only experiment conducted on human was small pilot study on patients with recurrent glioblastoma multiforme. THC was administrated intracranially directly into the tumor mass. It has been reported that this method was safe and no side effect was reported. In some patients temporal decrease of tumor growing was observed and activation of molecular mechanisms involved in apoptosis and in inhibition of proliferation of cancer cells were reported in two patients (Guzmán *et al*. 2006).

That study was too small to draw significant conclusions, but it shows a need to conduct subsequent studies in that field. It is necessary to assess

optimal patients selection, administration routes or interaction with other drugs.

Recently two safety clinical trials were conducted in human. In the first, Sativex in combination with temozolomide were studied in patients with glioblastoma multiforme and in the second, CBD for acute graft-versus-host disease in patients who have undergone allogeneic hematopoietic stem cell transplantation (ClinicalTrials.gov 2016a, ClinicalTrials.gov 2016b).

On the other hand the role of endocannabinoid system in carcinogenesis is still unclear. It has been shown that level of endocannabinoids and expression of cannabinoid receptors are elevated in many cancers, moreover, that seems to be correlated with the degree of malignancy (Guzman 2003). Increased concentrations of AEA and 2-AG were observed in cases of glioblastoma, prostate adenocarcinoma, colon cancer and pituitary adenoma (Pisanti *et al*. 2013). Elevated expression of CB1 receptor was demonstrated in ovary and colon cancers and in hepatocellular carcinoma (Messalli *et al*. 2014, Mukhopadhyay *et al*. 2015, Park 2012). Increase in expression of CB2, in turn, was observed in breast cancers, gliomas and astrocytoma (Caffarel *et al*. 2006, Sánchez *et al*. 2001). Interestingly, it has been shown that CB receptors at least partially mediate in the development of skin cancer induced by UV irradiation (Zheng *et al*. 2008). Mice devoid of CB receptors showed marked decrease in UV-induced carcinogenesis. Similar results were obtained in hepatocellular cancer model – inactivation of CB1 receptor led to the suppression of hepatocarcinogenesis (Suk *et al*. 2016). Another important observation is that pharmacological blockage of CB1 by its antagonist leads to decrease in carcinogenesis in some models (Marshall *et al*. 2011, Mukhopadhyay *et al*. 2015, Pisanti *et al*. 2011, Sarnataro *et al*. 2006).

There are studies showing that cannabinoids can stimulate proliferation of cancer cells in some circumstances. In cases of glioblastoma and lung carcinoma cells incubated with nanomolar concentrations of THC, cell proliferation was accelerated. This phenomenon was based on activation of epidermal growth factor receptor (EGFR) and downstream activation of ERK1/2 pathway (Hart *et al*. 2004). Systemic administration of THC has been shown to increase tumor size and number of metastasis in murine model (Mckallip *et al*. 2005). Cannabinoids interacting with CB2 receptor act as immunosuppressants. This probably leads to suppression of antitumor immune response by THC, stimulating development of tumor. FAAH-deficient mice with elevated level of AEA showed increase in hepatocarcinogenesis (Suk *et al*. 2016).

Conclusions

Despite some important gaps in the knowledge of cannabinoids' impact on cancer cells, use of cannabis and cannabinoids-based medicines in anticancer treatment raises big hopes. Especially applying a combination of classical chemotherapy and pharmacological stimulation of endocannabinoids system could be very promising.

However, it is still too early to admit the use of cannabinoid-based medicines as efficient and safe. There is a lack in studies concerning safety on cannabinoids in treatment and their potential interactions with other drugs. Especially, concerns can be raised by studies showing that in some cases activation of CB receptors can promote development of cancer. We still do not

fully understand specific role of particular elements of endocannabinoid system in carcinogenesis. There is a need to ascertain a specific instances in which the use of cannabinoids can be considered as safe. Another important point is that all studies of anticancer characteristic of cannabinoids were conducted *in vitro* and in animal models. Reliable, well-prepared clinical trials are needed to assess the true efficacy, safety and implications of cannabinoids in cancer treatment.

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Streszczenie

Kannabinoidy oddziałują na organizm ludzki wiążąc się z receptorami kannabinoidowymi (CB1 oraz CB2). Dwoma głównymi kannabinoidami roślinnymi są Δ^9-tetrahydrokannabinol (THC) i kannabidiol (CBD). THC wiąże się z receptorami CB1 obecnymi w obrębie centralnego układu nerwowego, co powoduje psychoaktywne właściwości marihuany. CBD posiada niskie powinowactwo do receptorów CB1, nie posiada właściwości psychoaktywnych, co sprawia, że jego medyczne zastosowanie może być znacznie szersze. Receptory CB są częścią złożonego mechanizmu zaangażowanego w regulację wielu procesów fizjologicznych – układu

endokannabinoidowego. Kannabinoidy znalazły pewne zastosowanie w medycynie paliatywnej, lecz istnieje wiele badań dowodzących ich antynowotworowych właściwości. Agoniści receptorów CB1 powodują akumulację związków z grupy ceramidów w komórkach nowotworowych, stres retikulum endoplazmatycznego i w konsekwencji apoptozę. W efektach wywoływanych przez kannabinoidy posiadając niskie powinowactwo do receptorów CB pośredniczy najprawdopodobniej indukcja produkcji reaktywnych form tlenu. Dotychczasowa wiedza dotycząca przeciwnowotworowych właściwości kannabinoidów opiera się tylko na badaniach przedklinicznych. Istnieje potrzeba przeprowadzania kolejnych badań, które umożliwiłyby oszacowanie rzeczywistego potencjału tych związków.

Folia Biologica et Oecologica 12: 26–37 (2016)
Acta Universitatis Lodziensis

Methods for eradication of the biofilms formed by opportunistic pathogens using novel techniques – A review

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ABSTRACT

The inconvenient environmental conditions force microorganisms to colonize either abiotic surfaces or animal and plant tissues and, therefore, form more resistant structures – biofilms. The phenomenon of microbial adherence, opportunistic pathogens in particular, is of a great concern. Colonization of medical devices and biofilm formation on their surface, may lead to severe infections mainly in humans with impaired immune system. Although, current research consider various methods for prevention of microbial biofilms formation, still, once a biofilm is formed, its elimination is almost impossible. This study focuses on the overview of novel methods applied for eradication of mature opportunistic pathogens' biofilms. Among various techniques the following: cold plasma, electric field, ultrasounds, ozonated water treatment, phagotherapy, matrix targeting enzymes, bacteriocins, synthetic chemicals and natural origin compounds used for biofilm matrix disruption were briefly described.

KEY WORDS: biofilm eradication, *Pseudomonas aeruginosa*, microbial colonization

Introduction

Opportunistic pathogens and the risk they carry

According to a definition, opportunistic pathogens are the organisms which are able to cause disease only when the host's resistance is impaired by other diseases, genetic defects, medical procedures, drugs therapies or age (for example AIDS, cystic fibrosis, chemotherapy, immunosuppression). They are not highly virulent in contrary to true pathogens, that through production of virulence factors may simply evade host defences and harm host tissues (Relman & Falkow 1990).

The conception of opportunistic pathogens is strictly linked to healthcare-associated infections (HAI) as the patients are the most exposed group.

DOI: 10.1515/fobio-2016-0003

According to the European Centre for Disease Prevention and Control (2012), the total number of long term-care-associated infections in EU each year was estimated at 4,3 million. In addition, it was also evaluated that 4,1 million patients acquired the HAI in acute-care facilities. Regarding infection connected with ICU (Intensive Care Unit) the most common among: blood stream infections were caused by coagulase-negative staphylococci, *Enterococcus* spp., *Staphylococcus aureus*; urinary tract infections were *Escherichia coli, Candida* spp., *Enterococcus* spp.; pneumonia cases were *Pseudomonas aeruginosa, S. aureus, E. coli* (Fig.1). All the mentioned microorganisms are supposed to be opportunistic (Annual Epidemiological Report, 2012).

Attempts to remove those microorganisms often fail as they are capable of colonizing medical devices such as catheters, tubes, stents, needles, implants etc. and form a complex structure on these surfaces called biofilm (Zabielska *et al.* 2015).

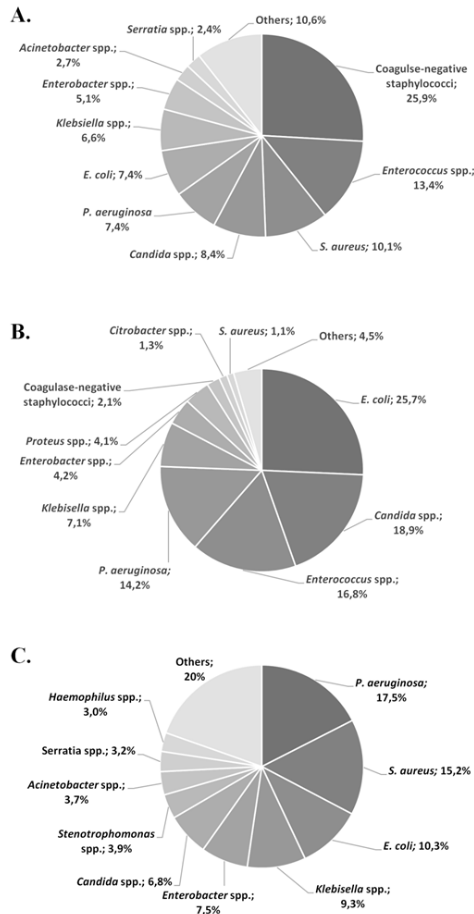

Figure 1. Percentage content of microbial infections associated with blood (A), urinary tract (B) and lung (C), (based on Annual Epidemiological Report, 2012).

Biofilm characteristics and formation

Biofilms are regarded as dynamic structures of microbial communities of either one or several species enmeshed within extracellular matrix and adhered (classic definition) to biological or abiotic surfaces. Microbial biofilms are also considered as a manner to survive inconvenient environmental condition, as it is reported that cells in a form of the biofilm are more resistant than planktonic ones (Garrett *et al.* 2008). Moreover, researches claim that bacteria embedded in extracellular polymeric substances (EPS) express higher tolerance to antibiotics, disinfectants and are harder to remove from surfaces (Donlan 2001, Furowicz *et al.* 2010, Stewert & Costerton 2001). Biofilms undergo constant changes within their composition, both chemical and biological. External matrix provides suitable conditions for adherence of other microorganisms and, therefore, diversification of biofilms' microbiota.

Formation of biofilm is a complex process which depends on various environmental factors (surface porosity, fluids flow, nutrients availability, etc.) and could be divided into four major steps (Garrett *et al.* 2008). The initial step, in which free-swimming microbial cells attach to the particular biotic or abiotic surfaces, is reversible (Fig.2, A). Planktonic cells can migrate towards the surface of biomaterial by means of physical forces (e.g. van der Waals forces), fluids flow (passive cell transportation) or using their flagella and fimbria (Kolwzan 2011, Haiko & Westerlund-Wikstrom, 2013). At this early stage, single adhered cells do not form a stable structure and, therefore, could be easily removed from the material surface with physical or chemical methods. Whether cells attachment is not affected by any external disruption, the irreversible phase of biofilm formation occurs (Fig. 2, B). The subsequent cell proliferation and production of extracellular polymeric substances (EPS) enables creation of microcolonies enmeshed within biopolymeric matrix (Donlan 2001). The surrounding slime matrix consist of various substances which content differs among microbial species. Nevertheless, major contribution in the EPS composition derives from water and polysaccharides (Czaczyk & Myszka 2007).

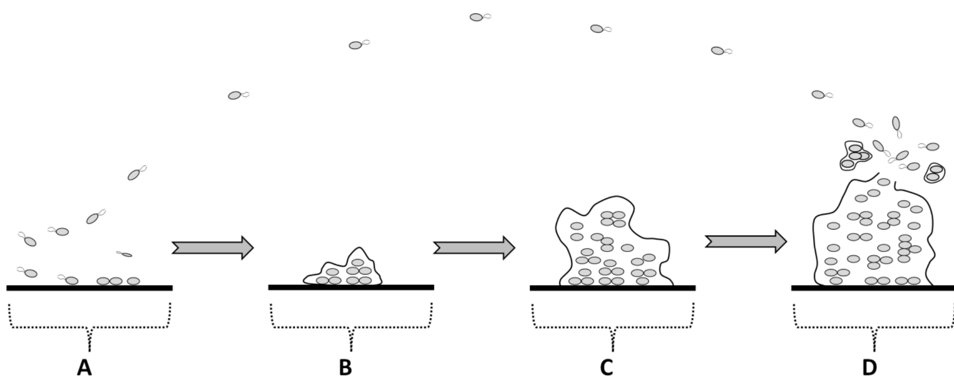

Figure 2. Mechanism of biofilm formation: A – single cells attachment and reversible adhesion; B – EPS production, microcolony formation and irreversible adhesion; C – biofilm maturation; D – microbial cells/aggregates dispersal (based on Donlan, 2001; Kolwzan 2011, Maciejewska *et al.* 2016).

The presence of extracellular polymeric substances is pivotal for biofilm functioning. Within the matrix, cells differentiate, form microcolonies, change their metabolism and gradually specialize their functions, therefore, mature biofilms consist of multilayer system (Fig. 2, C). The cells in outer-layer remain active, proliferate and continuously secrete metabolic products. The deeper-laying cells are subjected to limited oxygen and nutrient inflow, thus their metabolism alters toward activation of anaerobic metabolic pathways and inactivation of some enzymes synthesis (Kolwzan 2011). As a result, cells embedded inside the biofilm exhibit different features than planktonic cells.

Biofilm cells differentiation and metabolic activity is associated with signal transduction phenomenon called quorum sensing (QS). QS is a way of communication based on the production of autoinducers (chemical signals) and receptors (proteins receiving signals) which pass from cell to cell (Myszka & Czaczyk 2010). Quorum sensing, thus the cell's communication is, however, facilitated within the biofilm because of microbial density. Accumulation of particular autoinducers 'inform' microbial community about density of their cells and thus helps to maintain proper biofilm regulation. The mechanism of signal transmission is different for Gram-negative and Gram-positive bacteria (Miller & Bassler 2001, Myszka & Czaczyk 2010, Kolwzan 2011). Gram-negative bacteria predominately communicate with acyl homoserine lactone signaling molecules (AHLs). The general structure of AHLs is universal, however, the kind of a substituent incorporated in the α-position is specific for microbial species (Myszka & Czaczyk 2010). On the contrary, Gram-positive bacteria use oligopeptides which are not able to diffuse freely outside the cytoplasmic membrane and should be excreted outside the cell by ATP-dependent transporter proteins (Miller & Bassler 2001, Kolwzan 2011). Although there are specific communication pathways for particular microbial species, there exist a group of universal signal chemicals, called autoinducer-2 (AI-2) molecules, which might enable communication between different microorganisms (Kolwzan 2011).

Signaling pathways of biofilm communities provide proper functioning of this structure. Formation of thick mature biofilm together with accumulation of signal molecules inducers may lead to the disruption of biofilm matrix and the release of single cells or small aggregates. In a consequence, the dispersion of liberated cells enables their propagation among the environment and further colonization of other surfaces (Fig. 2, D). Moreover, quorum sensing might promote particular genes expression which are responsible for antibiotic resistance and anti-drug control (Maciejewska *et al.* 2016). Additionally, the biofilm's EPS coating prevents chemical molecules to enter inside the biofilm structure and act directly onto microbial cells. Therefore, once an irreversible stage of biofilm is achieved, its elimination is hard to obtain or ever impossible. New methods for fighting against biofilms aim to either early stage of biofilm development (reversible adhesion), modification of biomaterials' surfaces or disruption of mature biofilm matrix (Cortez *et al.* 2011, Chen *et al.* 2013).

Methods for bacterial biofilms eradication

Physical methods

Since the biofilm structure disposal form surfaces via chemical substances has been well studied, still the easiest way for its elimination seem physical

procedures like e.g. scrapping. However, it is claimed that scrapping is not effective enough due to variety of materials' structures. Another common technique is thermal processing, both in high and low temperatures. Over one-hour exposure in temperature of 95°C significantly reduces the level of microbial biofilms. Similar effects were reported for multiple freezing procedure (Maciejewska *et al*. 2016).

A very promising approach in the process of biofilm eradication seems to be an electromagnetic field. It is reported that Pulsed Electric Fields (PEF) disrupt biofilm matrix of *P. aeruginosa* formed on medical implants (Khan *et al.* 2016). Still the authors suggest that PEF combined with antibiotics may stimulate human immune system and, however, further test involving *in vivo* models should be considered.

The newest researches consider usage of low-temperature (cold) atmospheric pressure plasma for decontamination of surfaces and elimination of bacterial biofilms. A cold plasma treatment with addition of electrospraying against *E. coli* biofilm was studied (Kovalova *et al.* 2016). It was found, that 15-minute exposure to the corona discharge leads to detachment of partial biofilm matrix and the remaining biomass has decreased by 53.6-66.3%. The addition of the water electrospray resulted in more intense *E. coli* biofilm matrix detachment (63.5-70.5% decrease). Similar studies were proceeded by Ziuzina *et al.* (2015), however, not only on *E. coli* biofilm eradication, but also on *P. aeruginosa* virulence testing. The viability of *E. coli* biofilms subjected to a direct and indirect atmospheric cold plasma treatment (ACP) decreased by around 4 log units after 60s exposure. In addition, the metabolic activity of 48-hour *E. coli* biofilm was reduced by about 78% for both direct and indirect ACP exposure. Moreover, the examination of cold plasma treatment applied to *P. aeruginosa* biofilms revealed that ACP acts effectively on two virulence factors of these bacteria – pyocyanin and elastase production. However, the reduction in their concentration did not affect the viability of formed biofilm (Ziuzina *et al.* 2015). On the contrary, the studies conducted by Alkawareek *et al.* (2012) and Ziuzina *et al.* (2014), showed that extended ACP treatment has a significant impact on viability and metabolic activity of *P. aeruginosa* planktonic cells and biofilm matrix.

Physico-chemical methods

Ronan *et al.* (2016) have studied the effect of antibiotics (gentamicin or streptomycin) combined with ultrasound and microbubbles (USMB gas-filled microstructures encapsulated by lipid, polymer shell or proteins) treatment against *P. aeruginosa*. Application of USMB, gentamicin or streptomycin alone did not affect the biofilm structure in a great extent. The ultrasounds and microbubbles injection followed by the exposure to antibiotics, resulted in changes in *P. aeruginosa* biofilm matrix and significantly reduced its respiration rate.

The potential anti-biofilm activity was observed for ozonated water as well. The research conducted by Bialoszewski *et al.* (2011) indicates that even 30s exposure of *S. aureus* biofilm to freshly ozonated water results in significant reduction of cells viability. On the contrary, *P. aeruginosa* early stage biofilm expressed higher tolerance, however, mature biofilms (48 and 72-hour biofilms) appeared to be more susceptible to ozonated water. In different study, Hanley-Onken & Cohen (2013) have tested the impact of ozonated water sterilization protocol

against *E. coli* biofilm formed on the stainless steel surface. It was observed that this treatment provides effective biofilm removal and can be used as alternative method for surface sterilization.

Another alternative seems to be photodynamic therapy (PDT) which involves usage of a specific photoactive dye and its activation after an exposure to particular light wavelength (Konopka & Goslinski 2007, Maciejewska 2016). PDT was found to be appropriate as antimicrobial therapy against both drug-resistant microorganisms and biofilms (Hamblin & Hasan 2004, Konopka & Goslinski 2007). Biel *et al.* (2011) reported, that antimicrobial photodynamic therapy tested *in vitro* is effective against planktonic cells and biofilm of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA). The reduction of both bacteria reached 99.9% after a single treatment. Similar results for both planktonic cells and biofilm were obtained by Street *et al.* (2008). The treatment of free-swimming *P. aeruginosa* cells by means of photodynamic disinfection resulted in more than 7 log units reduction in cell number, whereas 24-hour biofilm was eradicated in 99.0% and 99.9% for single and double exposure respectively.

Chemical compounds

Bacteria in biofilm matrix are reported to be less sensitive than planktonic forms towards variety of chemical antimicrobials such as antibiotics, disinfectants and their minimal inhibitory concentration (MICs) are even thousands times higher for biofilm. Many mechanisms are considered to be responsible for biofilm resistance to chemicals. Exopolysaccharides seem to be the main reason as they limit diffusion in the biofilm interior and increase the number of free functional groups. Additionally, slow antimicrobials penetration into further biofilm layers may result in their inactivation by microbial enzymes or removal via efflux pumps. Also the presence of super-resistant cells in deeper layers of biofilm, due to their lack of metabolic activity, weakens the effect of antimicrobials (Sen *et al.* 2015, Kolwzan 2011, Myszka & Czaczyk 2007).

Kwiecinska-Pirog *et al.* (2016) have tested the impact of ciprofloxacin on biofilm formation by *Proteus mirabilis* and *Proteus vulgaris* clinical strains. Ciprofloxacin belongs to 2nd generation quinolones and is considered as the strongest among them. They proved that ciprofloxacin at concentration of 0.06 μg/ml may have been efficient against some strains (reduction over 50%), especially against *P. vulgaris*.

Combination of gentamicin and L-arginine against *S. aureus, E. coli* and *P. aeruginosa* single-strain biofilms were examined by Lebeaux *et al.* (2014). It was found that the addition of L-arginine increased bacteria susceptibility to gentamicin and led to almost complete biofilm eradication at the gentamicin concentration of 200×MIC.

In the research presented by Rosenblatt *et al.* (2015) the synergistic effect of caprilic acid and glyceryl trinitrate (GTN) against MRSA, MRSE (methicillin-resistant *Staphylococcus epidermidis*) and multidrug-resistant *P. aeruginosa* was evaluated. The combination of 0.05% caprylic acid, 0.04% GTN and 5.0% dextrose was very efficient and the biofilm reduction on silicone discs was close to 100% after 2-hour exposure.

Among the recent research an approach of Qu *et al.* (2016) using norspermidine (polyamine) to eradicate *P. aeruginosa* biofilm is noteworthy. The results indicate that norspermidine at concentration of 10 mmol/L can either

prevent from microbial cell attachment to surfaces or disassemble 24-hour mature biofilm with a great efficiency (even 80-90%). This substance also decreases quorum sensing genes expression, pyocyanin production and enzymes activity (elastase, protease).

The other method involves achievements of nanotechnology is usage of nano-penicillin G (Fernandes *et al.* 2016). They obtained nano/micro-sized, oil-filled, surfactant-containing spheres which were able to interact with the membrane of Gram-negative bacteria. Just the presence of surfactant together with penicillin G is crucial for efficient penetration. After *P. aeruginosa* and *E. coli* biofilm contact with nano-penicillin G, they quantified the amount of viable bacteria within biofilms. It was reported that *P. aeruginosa* was more sensitive to the nano-antibiotic than *E. coli*. Similarly penicillin G was used in solution which, in contrast to nano-penicillin G, appeared to be not effective at all against *P. aeruginosa* and induced a 0.8 log CFU/ml reduction of *E. coli* biofilms.

Nanoparticles were also used by Ahmed *et al*. (2016). They treated *Klebsiella penumoniae* biofilm with gold nanoparticles conjugated with chlorchexidine (Au-CHX). A significant biofilm disruption of the tested isolates for Au-CHX at concentration of 100 µM was achieved, whilst non conjugated chlorchexidine even at the concentration of 2 mM was not effective. It was suggested that nanoparticles might have contacted with hard-to-reach bacteria in internal layers of biofilm through water channels formed within biofilm structure.

Natural compounds and phages

Currently researchers express a great interest in the use of natural origin substances e.g. essential oils and their constituents. Due to their unique composition and action simultaneously focusing on different targets in a cell, plant derivatives remain effective antimicrobials. Moreover, their usage in combination with antibiotics may exude synergistic effects. The effect of natural substances on microorganisms is multidirectional and includes, inter alia, β-lactamase inhibition, bacterial efflux pump inhibition, cell wall and membrane disturbances and anti-quorum sensing activity (Yap *et al.* 2014).

Anti-biofilm activity of *Mentha pulegium* (Pennyroyal) against multidrug-resistant *Acinetobacter baumanii* was reported by Tutar *et al.* (2016). *M. pulegium* essential oil expressed a strong antimicrobial activity and was able to eradicate biofilm even at ½ MIC concentration. The best results were obtained for this oil at MIC concentration and the reduction in biofilm formation reached 80-90%. Biofilm metabolic activity was also remarkably inhibited at the 2.5 µl/ml essential oil concentration.

The initial attempts involving *S. aureus* biofilm formation and control on stainless steel by component of oregano and thyme essential oil, carvacrol, were proceeded by Knowles & Roller (2001). Combining carvacrol, eugenol and mild micellar surfactants successfully inhibited the growth of *E. coli* O157:H7 and *Listeria monocytogenes* (Perez-Conesa *et al.* 2006). The approach of Yadav *et al.* (2015) based on the effect of eugenol against *S. aureus* was also examined. Eugenol is a major component of clove oil with wide application in food and cosmetic industries due to its antimicrobial, antioxidant, anti-inflammatory, anticarminative and antispasmodic activity. The biomasses of established biofilms of MRSA and MSSA (methicilin-sensitive *Staphylococcus aureus*) were

significantly decreased and their eradication reached the level of 80-90% (0.08% eugenol solution – 2×MIC). The obtained results indicated that eugenol anti-biofilm activity may be due to the disruption of the cell-to-cell connections and cell lysis.

Some of natural compounds express high cytotoxicity, e.g. tea tree oil. Despite good antimicrobial activity *in vitro* their application *in vivo* very often is impossible, since effective concentration is cytotoxic for eukaryotic cells (Hammer *et al.* 2006).

The effect of green tea compound epigallocatechin-3-gallate (EGCg) against *Strenotrophomonas maltophilia* biofilm was evaluated by Vidigal *et al.* (2014). 24-hour and 7-day biofilms after 24-hour exposure to the EGCg were decreased in comparison to untreated biofilms. It is assumed that ECG is capable of binding and damaging bacterial membranes. The antibiofilm effect of green tea was not so spectacular, however it consumed as a beverage or inhaled as a green tea extract solution may serve as a safe agent for intestinal or upper respiratory tract biofilm inhibitor, respectively.

Different group of natural substances are biosurfactants, surface-active substances produced by microorganisms with anti-adhesive and biofilm disruption capabilities.

A novel approach was presented by Diaz De Rienzo *et al.* (2016) who used rhamnolipids and combination of rhamnolipids and caprylic acid against *P. aeruginosa* biofilm. The highest impact on mature biofilm was observed for the mixture of rhamnolipids and caprylic acid (biofilm reduction over 60%). It was found that rhamnolipids may interfere with cell-to-cell interactions and cell-substratum interactions as well.

Nowadays, a particular interest should be paid to novel biological methods in treatment of bacterial biofilms. Apart from the natural substances like plant metabolites or essential oils components, researches considered biofilm eradication with matrix targeting enzymes (Thallinger *et al.* 2013). The enzymes applied cause degradation of biofilm matrix by disruption of extracellular polymeric substances, thus eDNA, proteins and polysaccharides (Chen *et al.* 2013). *In vitro* studies showed that staphylococcal and enterococcal biofilms might be disrupted by N-acetyl-D-glucosamine-1-phosphate acetyl transferase. Similarly, biofilm formed by *S. aureus* was dispersed when treated with proteinase K or trypsin, whereas *S. epidermidis* biofilm matrix was disrupted after dispersin B application (Kaplan *et al.* 2004, Chaignon *et al.* 2007).

Treatment of biofilms with natural microbial substances, bacteriocins, seems to be promising as well. Bacteriocins are considered as protein substances excreted by both Gram-positive and Gram-negative bacteria which aim to inhibit or kill other microorganisms. The effect of three bacteriocins (nisin A, lacticin Q, and nukacin ISK-1) against MRSA was evaluated (Okuda *et al.* 2013). Among three tested substances, bactericidal ability on *S. aureus* biofilms was observed only for nisin A and lacticin Q.

An emerging interest could be find in biofilms elimination by usage of lytic bacteriophages (Carson *et al.* 2010). Great variety of phages has been reported to encode enzymes capable of EPS degradation (Hughes *et al.* 1998). Sharp *et al.* (2010) has described the ability of phages to penetrate through the EPS layer and infect *P. aeruginosa* cells with their polysaccharide lytic enzymes. On the other hand, Carson *et al.* (2010) have studied the effect of bacteriophages on *P.*

mirabilis and *E. coli* established biofilms. It was found that phage treatment has reduced the biofilm populations by almost four log units. Further study on biofilms formed on the surface of catheters previously impregnated with hydrogel and exposed to lytic bacteriophages (*E. coli* T4 phage and coli-proteic bacteriophage) showed almost 90% extinction in both *E.coli* and *P. mirabilis* biofilms (Carson *et al.* 2010). The research conducted by Nouraldin *et al.* (2015) concerning concurrent phages and antibiotics application suggests that both planktonic cells and *P. aeruginosa* biofilms are less susceptible when using antibiotics or phages alone. The antibiotic-phage combination expressed a synergistic effect in *P. aeruginosa* biofilm eradication.

Conclusion

Microbial biofilm is a structure which constantly surprises researchers with its complexity and the mechanisms of development. As the resistance of microorganisms in biofilm can be extremely high, it is crucial to find an effective way to stop the process of biofilm formation or once the biofilm is established, to remove it.

Elimination of biofilm is significant in the clinical environment as opportunistic pathogens colonizing medical equipment may pose a threat for patients with impaired immune system, leading to serious diseases and consequently to death. What is more, it has to be considered that the biofilms may develop on biotic surfaces as well, such as pulmonary epithelium.

The most promising therapies for biofilms eradication seem to be combining gold nanoparticles with antibiotics or antibiotic in the form of nanoparticles, which are able to penetrate deeper layers of biofilm and destroy its internal structure.

Also natural origin substances deserve to be highlighted. Except their ability to eradicate biofilm with a great efficiency there was no increase in microbial resistance after prolonged contact with these specific antimicrobials. Moreover, such compounds may be usually used as food-additives, cosmetic compounds and pharmaceutical products.

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Streszczenie

W niekorzystnych warunkach środowiska, mikroorganizmy zasiedlają zarówno powierzchnie abiotyczne, jak i biotyczne takie jak tkanki zwierzęce czy roślinne, tworząc struktury biofilmu charakteryzujące się wysoką opornością. Adhezja mikroorganizmów, szczególnie patogenów oportunistycznych, niesie niebezpieczeństwo zasiedlania materiałów medycznych, co może doprowadzić do infekcji u osób z obniżoną odpornością. Chociaż dotychczasowe badania wskazują różne metody zapobiegania tworzeniu biofilmu, jego całkowita eliminacja ze środowiska jest nadal niemożliwa. Przedstawione opracowanie stanowi przegląd

nowoczesnych metod usuwania dojrzałego biofilmu tworzonego przez patogeny oportunistyczne. Spośród wielu metod opisano m.in. zastosowanie: zimnej plazmy, ultradźwięków, pola elektrycznego, ozonowania wody, terapii fagowej, enzymów działających bezpośrednio na macierz biofilmu, bakteriocyn, środków chemicznych syntetycznych oraz pochodzenia naturalnego.

Folia Biologica et Oecologica 12: 38–47 (2016)
Acta Universitatis Lodziensis

Adipose-derived stem cells: a review of osteogenesis differentiation

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ABSTRACT

This review article provides an overview on adipose-derived stem cells (ADSCs) for implications in bone tissue regeneration.
Firstly this article focuses on mesenchymal stem cells (MSCs) which are object of interest in regenerative medicine. Stem cells have unlimited potential for self-renewal and develop into various cell types. They are used for many therapies such as bone tissue regeneration. Adipose tissue is one of the main sources of mesenchymal stem cells (MSCs). Regenerative medicine intends to differentiate ADSC along specific lineage pathways to effect repair of damaged or failing organs. For further clinical applications it is necessary to understand mechanisms involved in ADSCs proliferation and differentiation.
Second part of manuscript based on osteogenesis differentiation of stem cells. Bones are highly regenerative organs but there are still many problems with therapy of large bone defects. Sometimes there is necessary to make a replacement or expansion new bone tissue. Stem cells might be a good solution for this especially ADSCs which manage differentiate into osteoblast in *in vitro* and *in vivo* conditions.

KEY WORDS: mesenchymal stem cells, regenerative medicine, adipose tissue

Mesenchymal stem cells

Mesenchymal stem cell (MSCs) are non-hemotopoetic, multipotentent, adult stem cell. MSCs have ability to self-renew and differentiate into multiple tissues, including bone, cartilage, fat, and other tissues of mesodermal origin. They are present in blood, adipose tissue, bone, skin and Wharton's jelly (Maleki et al. 2014).

The multidirectional therapeutic potential of MSCs has generated increasing amount of research in all over the world. It caused lack of homogenous methods in isolation, cell culture and

DOI: 10.1515/fobio-2016-0004

identification of mesenchymal stem cells. It has forced The International Society for Cellular Therapy (ISCT) and International Federation for Adipose Therapeutics (IFATS) to creating a minimal criteria to define MSCs. Based on it human MSCs identified by adherence to plastic and expression of cell surface markers including CD90, CD73, CD105 and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface proteins. There also must differentiate to osteoblasts, adipocytes and chondroblasts in vitro condition (Dominici et al. 2006, Fathi et al. 2016, Bourin 2013)(Tab. 1).

Table 1. Minimal criteria for defining mesenchymal stem cells.

Feature	
cell culture	adherence to plastic
differentiation potential	osteoblasts, adipocytes and chondroblasts
≥ 95% population of cell with expression surface markers	CD90, CD73, CD105
≤ 2 % population of cell with lack of surface expression	CD45, CD34, CD14 or CD11b, CD79a, CD19, HLA-DR

It was proven that MSCs have inflammatory, immunomodulatory functions and they can penetrate into inflammatory sites. They secrete factors such as: TGF- β , IL-6, -7, -8, -10, 11,-12, -14, inducible nitric oxide synthase (iNOS) and hemooxygenase (HO). MSCs can modulate immunological responses through T-cell-mediated (Takano et al. 2014, Rahimzadeh et al. 2014) They release also multiple angiogenic and growth factors: VEGF, HGH or IGH-1 (Fathi et al. 2016, Soulnier et al. 2010). It suggest that they are improving neovascularization and promote angiogenesis process (Abudusaimi et al. 2011).

Thus MSCs have been applied in various diseases connected with bone damages, for example: rheumatoid arthritis (Takano et al. 2014), avascular necrosis of the femoral head (Abudusaimi et al.2011) and large mechanical defects.

Adipose derived stem cel

Adipose tissue is one of the most richness source of stem cells. Adipose derived stem cells (ADSCs) are plastic-adherent cells, which are characterized by a variety of cell surface markers (Undale et al. 2009). They were first described. in 2001 as a population of cells derived from adipose tissue with the potential of differentiation (Zuk et al. 2001). Isolation method based on digested it with collagenase Type I, and separated the cellular components by centrifugation (Gimble et al. 2003). The number of cells after isolation is connected with amount of adipose tissue. The lowest amount of tissue is 0,1-2mg but the number of stem cells depend on tissue and their volume is associated with destination of ADSCs (Cheng et al. 2011).

ADSCs are able to differentiate into a number of mesenchymal cell types, including osteoblasts, chondrocytes and adipocytes (Undale et al. 2009, Fernandez et al. 2015).

Compared to other types of stem cells, ADSCs have many advantages. ADSCs can be easily obtained from a donor. Adipose tissue donation is the easiest and less invasive for patients in comparison e.g. bone marrow biopsy. Procedure of liposuction can provide a lot of tissue and cells. Moreover isolation of stem cells from bone marrow is less effective and cells often are contaminate (Chen at al.2013, Fathi et al. 2016, Dai et

al. 2016). Comparison of mesenchymal stem cells obtained from different tissues (fat tissue, bone marrow, placenta) showed that adipose stem cells did not differ morphologically from bone marrow cells. These cells have similar expression of the main marker genes (Musina et al. 2005).

It has been shown that from 1 gram of fat tissue may be isolated from 0.5 x 10^4 to 2 x 10^5 stem cells. Differences in the amount are connected with a gender, age, body mass index of donor but also medical record, type of adipose tissue (white or brown) and its location (Bajek et al. 2008, Olkowska et al. 2008). Research suggested that cells from younger donor are grown and differentiating better than from old donors (Bunnell et al. 2008, Musina et al. 2005).

Moreover there is not ethical problems with using ADSCs which is a huge issue for embryonal stem cell (Dai et al. 2016).

The transcriptome analysis with microarray technique of ADSC were reveals their more adipogenic potential than osteogenic in compared to bone marrow stem cells (BMSC). They also have larger capability to lipid synthesis. It suggest that ADSCs indicate better ability to differentiation into adipocytes than osteoblast. The differences were related to MSCs location. However ADSCs show lower immunogenicity than stem cells in bone marrow. Moreover tissue harvesting is easy, quick and efficient and thus they seem to be a better alternative as a stem cells source in compared another tissues (Bionaz et al. 2015, Monaco et al. 2012).

However, different methods of isolation of adipose tissue have influence on expression profile of genes characteristic of ADSCs. Comparison of adipose tissue collected during the surgery and adipose tissue collected by liposuction have shown the bigger amount of cell in case of liposuction and the population of these cells was more homogenous. As a result, processes of differentiation of both types of cells to mesoderm (cartilage, osteoblasts and adipocytes) it was more efficient in cells isolated from lipoaspirate than biopsy (Gnanasegaran et al. 2014). There is not many results about colleting stem cells from different adipose tissue places. It suggested that difference in number of cells between subcutaneous adipose tissue from the arms in compared to abdomen and breast. The amount of adipose derived stem cell is connected with location, type and species. (Kolaparthy et al. 2015).

The comparison of isolation adipose derived stem cells ADSC manual and automatic methods difference in cells activity did not observed. The number and viability of cells were similar in both cases (Doi et al. 2013).

Methods of differentiation of stem cells

Standard method for initiating osteogenic differentiation in stem cell culture is application some components which induce this process. Culture of MSCs in osteogenic medium causes manifestation of osteoblasts markers (Birmingham et al. 2012). Basic substances with proved action on osteogenesis in stem cells are: dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly). For osteogenic differentiation at least 21 days of treatment this substances are necessary (Langenbach et al.2013).

Mechanism of induces osteogenesis process by dexamethasone is multidirectional. Dex induces differentiation into osteoblast by activating Wnt/β-catenin pathways. It indicated that by Four And A Half LIM Domains 2 (FHL2) upregulation which influence on expression of *RUNX2*.

Consequently expression of collagen type I alpha 1 (*COL1A1*) is also upregulated. Moreover dexamethasone regulates the function of *RUNX2* via the activity of molecule TAZ which is transcriptional coactivator with PDZ-binding motif and mitogen-activated protein kinase (MAPK) phosphatase (MPK-1).

Bone morphogenetic protein (BMP) signaling also change the activity RUNX2 which was connected with initiating osteogenesis process. Binding BMPs to their receptors causes of phosphorylation SMAD proteins (SMAD 2, SMAD 5 and SMAD8) which subsequently bind with SMAD 4 and after translocation to nucleus regulates the expression of osteogenic transcription factors as RUNX2, OSX and DLX. Experiments showed that optimal concentration of dexamethasone in medium culture is 10nm (Langenbach et al. 2013).

Ascorbic acid induce differentiation of stem cells through the enhancement secretion of collagen type I into the extracellular matrix (ECM). It is a cofactor for hydroxylate proline and lysine which they are required for transformation pro-collagen into active form. β-glycerophosphate is a phosphate source in mineralization process and it induce expression of genes connected with osteogenesis through phosphorylation of kinases (Langenbach et al. 2013).

Numerus studies showed the relevant impact of vascular endothelial growth factor on the osteogenesis (Behr et al. 2011, Clark et al. 2015). VEGFA promotes the differentiation of progenitor cells into the direction of factors associated with angiogenesis, but also it impacts on the cells from bone tissue. VEGFA is one of the most important mediators of angiogenesis, cell migration and mineralization (Clark et al. 2015). It is extremely important element of osteogenesis due to bone vascularization during its expansion and repair (Behr et al. 2011). It was also noticed that the strong relationship occurs with VEGF factor and the bone morphogenetic proteins pathway. BMPs induces the intracellular signals which causes the differentiation of progenitor cells into osteoblasts (Zhang et al.2012). The treatment of ADSC with VEGF and BMP6 in vitro caused the increase in expression of alkaline phosphatase, genes associated with osteogenesis e.g. collagen type 1 (COL1A1), the osterix transcription factor and gene which encodes the homeotic protein DLX5 (distal-less homeobox 5) and also cells mineralization (Zhang et al.2012, Clark et al. 2015, Li and Madhu et al.2015, Li and Liu et al. 2015). Similar effect was observed both in the case of simultaneous treatment of ADSC with VEGF and BMP2, 4 and 9 (Zhang et al.2012, Li and Liu et al. 2015). Besides the use of VEGF and fibroblast growth factor (FGF-2) caused only the initiation of angiogenesis (Clark et al. 2015).

Experiments suggest also application of active form of 1,25(OH)2 D3 vitamin as the induction factor of osteogenesis (Kato et al. 2015). It was shown that there is possibility of generation both the osteoblasts and the cells similar to osteocytes from induced pluripotent cells inter alia by the supplementation with 1,25(OH)2 D3 vitamin (Kato et al. 2015).

Another factor showing the impact on differentiation of stem cells derived from adipose tissue is the hypoxia. It was noticed that the culture of ADSC in the conditions with the low level of oxygen (1-2%) strengthens the survivability and proliferation of the cells. This state intensifies the potential of differentiation into osteoblasts and also strengthens the expression of genes responsible for the maintenance of stemness: OCT4 (octamer-binding transcription factor 4),

NANOG (transcription factor), KLF4 (Kruppel-like factor 4) (Valorani et al. 2012, Xu et al. 2014).

The increase of genes associated with angiogenesis, adhesion and the growth factor releasement was also documented. Probably, it is the effect of physiological presence of low concentration of the oxygen at the stem cells niche (Valorani et al. 2012, Xu et al. 2014). Another study showed that the hypoxia weakens the proliferation ability of MSC but does not influences on the phenotype and seems to maintain them in the more immature stage than at the standard culture. This state caused the increase in pluripotent gene expression: SOX2 (SRY sex determining region Y-box 2), NANOG, OCT-4 (Ranera et al 2012).

The hormone participating in the regulation of glucose homeostasis in organism is the glucagon-like peptide type 1 (GLP-1). The use of GLP-1 in culture medium cause the increase in mRNA expression of markers specific to osteoblasts, the activity of alkaline phosphatase and mineralization of calcium. This hormone is especially important in insulin secretion by glucose-dependent pathway and has the anti-diabetic impact in the treatment of type 2 diabetes. Additionally, patients with diabetes have a higher risk for bone fracture and osteoporosis, which proves about close relationship which osteogenesis and the activity of GLP-1 hormone and the disruption of carbohydrate economy (Lee et al. 2015).

Estrogens acts also an important role at the formation of bone structure. The characteristic changes in the level of estrogens in the perimenopausal period causes osteoporosis, therefore numerus study suggest the use estrogens in the osteogenesis of the stem cells (Gao et al. 2015, Veronesi et al. 2015) (Tab. 2).

Table 2. Methods of differentiation of stem cells.

Differentiating factors	References
dexamethasone (Dex), ascorbic acid (Asc), β-glycerophosphate (β-Gly)	Langenbach et al.2013
vascular endothelial growth factor (VEGF)	Behr et al. 2011 Clark et al. 2015 Li and Madhu et al.2015 Zhang et al.2012
bone morphogenetic protein (BMP)	Zhang et al.2012 Clark et al. 2015 Li and Madhu et al.2015 Li and Liu et al. 2015
transforming growth factor (TGF-β)	Li and Liu et al. 2015
active form of 1,25(OH)2 D3 vitamin	Kato et al. 2015
hypoxia	Valorani et al. 2012 Xu et al. 2014 Ranera et al. 2012
glucagon-like peptide type 1 (GLP-1).	Lee et al. 2015
estrogens	Gao et al. 2015 Veronesi et al. 2015

Mechanism of osteogenesis stem cells

Treatment of large bone defects and incurable fractures is difficult clinical problem. Adipose tissue stem cells have ability to regenerate damaged bone tissue. However, there are necessary coexistence of efficient processes of angiogenesis and osteogenesis extending in order of their use (Behr et al. 2011).

The process of the osteogenesis of the stem cells derived from adipose tissue is regulated by the transcription of numerous genes. It should be noted, that in case of adipogenesis and osteogenesis the receptor proteins are activated by the PPAR (peroxisome proliferator-activated receptors). At the side of osteogenesis, these proteins acts as the negative regulator. The central role in this process is acted by the Wnt and PI3K/AKT and also the MAPK (mitogen-activated protein kinases) pathways (Bionaz et al. 2015, Chen and Shi et al. 2013). During the osteogenesis lots of growth factors such as bone morphogenic proteins (BMP), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF) and the vascular endothelial growth factor (VEGF) are secreted (Li and Madhu et al. 2015). The regulation of the majority of these factors is based on noncoding activity of micro RNA (miRNA) (Oshita et al. 2011, Chen et al. 2013).

The impact study of the interleukin family (IL-1) on the MSC confirmed its induction of the osteogenesis of human mesenchymal stem cells. It was proved that IL-1 activates the Wnt pathway i.e. the Wnt-5a gene and its orphan receptor of tyrosine-protein transmembrane receptor (ROR2). Similar effect was noted in the presence of cytokines such as interleukin-6 family (IL-6) and the tumor necrosis factor alpha (TNFα), besides with weaker effects of differentiation (Sonomoto et al. 2012,Tanaka 2015).

The differentiation of stem cells in vitro into the cells of bone tissue is multi-stage. In the first step, which is ongoing from five to fourteen days the expression of alkaline phosphatase (ALP) both at the level of transcriptome and proteome. ALP is known as reliable marker of early osteoblast differentiation (Clark et al. 2015). Additionally, at the early stage, the expression of collagen type 1 also increases and then the ALP level decreases. At the next stage which is ongoing from about fourteen to twenty-eight day increases the expression of osteocalcin an osteopontin (Birmingham et al. 2012)

To fully knowledge of differentiation mechanisms of stem cells into the osteoblasts will allow to maximize the use of this process in regenerative medicine.

3D culture of ADSC in regeneration of bone defects

ADSCs have shown promising results in many diseases. However positive results of experiments in two-dimensional plate culture are not meaningful, because of not sufficient condition of environment, without cell-cell and cell-environment interaction. The development of tissue and biomaterials engineering in last years resulted in a significant improvement of regenerative medicine and three-dimensional scaffolds are used more widely Three-dimensional cells culture techniques initiate cellular microenvironment similar to *in vivo*. 3D scaffolds have many advantages in compared to 2D culture. It observed that they can enhance the cell viability during proliferation (Dai et al.2016,)

Scaffolds are produced using biomaterials from selected components; they must be biocompatible and do not cause immune reaction. Their mechanical, chemical properties and microstructural patterns must be adapted to cell line. Scaffolds can be also biodegradable and non-biodegradable. It depends on its destination. Many studies indicate that ADSCs culture in 3D scaffolds can be alternative treatment in orthopaedic tissue repair (Dai et al. 2016). Nowadays traditional autologous and allogenous bone grafts are replacing

by different biomaterials. It is caused by lack of donor, potential disease transmission and severe immunogenic responses (Zhang et al. 2013).

Polylactic acid polymer scaffolds have the advantage of being degradable, porosity and easily moldable. It was found that polypyrrole-coated polylactide scaffolds can provide higher alkaline phosphatase (ALP) activity levels, which benefit the early osteogenic differentiation of ADSCs (Dai et al. 2016). Experiments show that PLA scaffolds escalate angiogenesis and osteogenesis of adipose derived stem cells but with co-culture with osteoblast or endothelial cell. It helps to create cell-cell interaction (Shah et al. 2014). These scaffolds provide properly growth and osteogenic differentiation of adipose derived stem cells (Lu et al. 2014). Chitosan is one of the substance which is examined for using in regenerative medicine of bone damage . It can be used as 2D or 3D scaffolds which have many advantages like: porosity, non-toxic and biocompatibility, high adsorption capacity and biodegradability (Busilacchi et al. 2013, Dash et al. 2011).

Conclusions

Adipose tissue is rich source of stem cells. ADSCs have multidirectional potential to differentiation inter alia into bone tissue. Development of regenerative medicine help in treatment large bone defects and their metabolism disorders. Unfortunately stem cells still must be examined for the safety of potential patients. Problems are inefficient differentiation, optimization of osteogenic medium and also comorbidities influence on proliferation and metabolism of stem cells.

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Streszczenie

Komórki macierzyste to komórki posiadające zdolność nieograniczonych podziałów oraz umiejętność do wielokierunkowego różnicowania. Mezenchymalne komórki macierzyste (MSC) to somatyczne komórki występujące w tkankach i narządach dorosłego organizmu takich jak: szpik kostny, tkanka tłuszczowa oraz mięśnie. Ulegają one różnicowaniu w kierunku komórek pochodzących z jednego listka zarodkowego jakim jest mezoderma. To pozwala na wykorzystanie ich w regeneracji chrząstki, kości lub wypełnienia ubytków tkanką tłuszczowa między innymi w chirurgi plastycznej.

Obecnie głównym źródłem z którego pozyskiwano MSC był szpik kostny, jednak coraz szersze zastosowanie wykazuje tkanka tłuszczowa. Komórki z niej pochodzące wykazują takie same właściwości jak te pochodzące z szpiku kostnego, a procedura izolacji jest dużo mniej inwazyjna dla pacjenta. Bardzo często natomiast ich ilość jest nieporównywanie większa. Stąd też niniejsza praca porusza temat wykorzystania MSC z tkanki tłuszczowej w regeneracji tkanki kostnej.

Folia Biologica et Oecologica 12: 48–59 (2016)
Acta Universitatis Lodziensis

The role of the Amyloid Precursor Protein mutations and PERK-dependent signaling pathways in the pathogenesis of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a highly complex, progressive, age-related neurodegenerative human disease entity. The genetic basis of AD is strictly connected with occurrence of mutations in *Amyloid Precursor (APP)* gene on chromosome 21. Molecular mechanism that leads to AD development still remains unclear. Recent data reported that it is closely correlated with Endoplasmic Reticulum (ER) stress conditions, which subsequently activate Unfolded Protein Response (UPR) signaling pathways, via the induction of protein kinase RNA-like endoplasmic reticulum kinase (PERK), as a self-protective, adaptive response to adverse stress conditions. That results in the attenuation of global protein synthesis and, on the contrary, selective translation of Activating Transcriptor Factor 4 (ATF4) and secretase β. Interestingly, under prolonged, severe ER stress UPR may switch its signal into apoptotic cell death. That ensues by ATF4-CHOP-mediated activation of a range of pro-apoptotic genes and, on the other hand, downregulation of the expression of the *anti-apoptotic protein B-cell lymphoma 2 (Bcl-2)* genes. Current investigations suggest that inhibitions of PERK activity may contribute to the attenuation of the deposition of toxic senile plaques in the brain tissue and, as a result, prevent degeneration of neurons and decline in cognitive abilities.

KEY WORDS: Amyloid β, Endoplasmic Reticulum stress, Unfolded Protein Response, eIF2α, CHOP

Introduction

Alzheimer's disease (AD) is a progressive, pathological, irreversible disease entity of the Central Nervous System (CNS) (Pedrini et al. 2009). AD, the most common type of dementia, constitutes as a huge health problem and has a significant influence on society. Current estimates suggest that nowadays 24 million people worldwide suffer from dementia (Pennanen et al. 2004). AD

DOI: 10.1515/fobio-2016-0005

with the highest frequency affect individuals in advanced age, but it is not a rigid rule, since AD may also affect younger people before the age 65 (Guerreiro et al. 2012, Babusikova et al. 2011). Type of AD, that tends to develops in individuals under 65 years is termed early-onset AD (EOAD), but in patients over 65 years is known as a late-onset AD (LOAD) (Tang & Gershon 2003). Interestingly, the number of patients with dementia is steadily increasing. Estimates suggest that AD may affect 65.7 million of people by 2030 and 115.4 million by 2050 (Babusikova et al. 2011).

AD is strictly connected with numerous changes not only in anatomy of tissue brain, but also in its biochemistry, genetic and function (Babusikova et al. 2011). The general features of AD are closely associated with memory loss and impairment of cognitive skills. AD and other neurodegenerative entities such as prion and Parkinson's diseases are connected with the accumulation of the misfolded or unfolded proteins in the lumen of the ER, which evoke ER stress conditions. That elicits activation of the PERK kinase and, as a consequence, Unfolded Protein Response pathways, that constitutes as a pro-adaptive cellular program to cope with unfavourable stress conditions. Paradoxically, long-termed stress conditions and over-activation of the PERK-dependent signaling pathways switch the pro-adaptive cellular response to pro-apoptotic signaling pathway (Moreno et al. 2012). Above-mentioned process leads to synaptic failure and significant loss of brain mass in AD patients (Pennanen et al. 2004). The main cause of Alzheimer's disease still remains unclear, but several lines of evidence suggest that the core of the problem lies in genetic disorder. Nowadays, available AD treatment is insufficient, since may only alleviate symptoms of AD. Due to that fact better understanding of the molecular mechanisms, that elicit cell death by apoptosis is a promising avenue on developing more effective AD treatments (Ballard et al. 2011).

Gene mutations and mechanisms involved in Aβ plaques aggregation

At the neuropathological level deposition of neurotoxic amyloid beta (Aβ) plaques in tissue brain as well as significant loss of neurons represent the main hallmark of AD (Kumar & Walter 2011). Senile plaques among the neurons in the brain are predominantly composed of Aβ peptide consisting of 39-42 amino acids, which is generated during Amyloid Precursor Protein (APP) processing. Longer form of Aβ consisting of 42 amino acids creates aggregates with higher frequency (Kumar & Walter 2011), since it is inherently more fibrillogenic as compared to the shorter form of Aβ40 (Price et al. 1995). Aβ40 is a predominant variant, which represent approximately 90% of all generated fragments of Aβ (Decock et al. 2016).

Numerous studies were undertaken to gather knowledge about the genetic basis of AD. *APP* on chromosome 21. is the first detected gene that is strictly connected with AD development (Tang & Gershon 2003). The product of the *APP* gene is one of the I transmembrane glycoprotein, that occurs in three different isoforms such as: APP695, APP751, APP770 amino acids (Belyaev et al. 2010). Proteolytic cleavage of the product of *APP* gene may occur via two different molecular pathways: amyloidogenic and non-amyloidogenic. During the first process a vital role plays

secretases β and γ, but during the second one α and γ (Ehehalt et al. 2003). Secretase β, also termed Beta-secretase 1 (BACE1), belongs to the family of aspartyl protease (Dislich & Lichtenthaler 2012) and, like APP, it is expressed in several areas of the brain. Interestingly, to confirm a fundamental role of BACE1 in AD pathogenesis scientists reported its increased level and enhanced activity in post mortem AD brains (Fukumoto et al. 2002, Harada et al. 2006). Furthermore, the level of secretase β and its biological activity are increased nearly twofold in AD tissue brain (Li et al. 2004). Secretase γ consists of a complex of proteins such as presenilin 1 (PS1) and presenilin 2 (PS2), nicastrin, anterior pharynx-defective 1 (Aph1) as well as presenilin enhancer 2 (Pen2) (Cole & Vassar 2007). The third one, secretase α, is the member of the ADAM 10 family of disintegrin metalloproteinase (Lichtenthaler 2012).

The physiological processing of APP occurs within the Aβ sequences, therefore precludes generation of full-length Aβ (Sisodia 1992). During this pathway first cleavage through secretase α, at the specific site K687/L688, releases two extracellular products such as N- terminal APPsα and C-terminal fragment C83. The second one is processed via secretase γ that leads to the production of two smaller, non-amyloidogenic fragments: p7 and p3 (Vassar 2004). Amyloidogenic APP processing via secretase β occurs generally at the specific site such as M671/D672 and G680/Y681 (Vassar et al. 2009). That generates amino-terminal fragment APPsβ as well as membrane-associated C99, that is proteolytically cleaved by secretase γ. After the second cleavage toxic Aβ peptide and amino-terminal fragment termed APP intracellular domain (AICD) is generated (Chow et al. 2010). Secretase γ is a specific enzyme that cleaves C99 fragment at sites that generates Aβ consisting of different number of amino acids such as: G708/G709 - Aβ37; G709/V710 – Aβ38; V711/I712 – Aβ40; V713/I714 - Aβ42 (Tian et al. 2010, Perez et al. 1999). Hence, secretase γ processing is fundamental for AD development, since it creates Aβ consisting of different number of amino acids, including its pathogenic, toxic form Aβ42 (Vassar et al. 2009) (Fig. 1).

Chromosome 21. is known as the smallest human autosome. Mutations in 14 known genes localized on chromosome 21. are known as a major cause of numerous monogenic disorders (Hattori et al. 2000). There is a body of evidence suggesting that APP mutations constitute as one of the main cause of EOAD as well as Familial Alzheimer's disease (FAD) with autosomal dominant inheritance. That leads to rapid changes in brains' neurons and, as a consequence, pathological cleavage of APP, which cause amyloidosis via extracellular deposition of Aβ plaques among the neurons (Weggen & Beher 2012). Moreover, above-mentioned *APP* mutations lead to the aberrant cleavage of APP via specific secretases (Hardy 1997). It has been reported that the most common APP mutations are the London, Dutch, Swedish and Flemish among others (Tang & Gershon 2003) (Tab. 1). Mutations in *APP* gene, which are localized at the cleavage site especially for secretase β promote APP processing in amyloidogenic pathway, but mutations near the cleavage site for secretase γ cause increased generation of Aβ42 with higher ability to creation of senile plaques in tissue brain (Zhou et al. 2011).

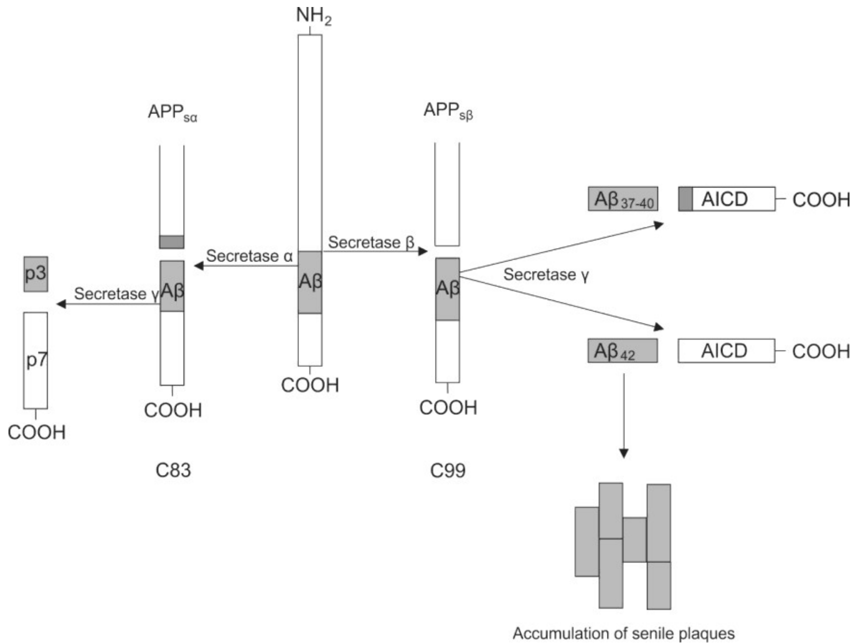

Figure 1. Molecular mechanisms of processing of the APP by α, β and γ secretases (Aβ – amyloid beta, APP – Amyloid Precursor Protein, APPsα - soluble Amyloid Precursor Protein-α, APPsβ - soluble Amyloid Precursor Protein-β, AICD - Amyloid Precursor Protein intracellular domain, C83 - 83-amino-acid C-terminal fragment, C99 – 99-amino-acid C-terminal fragment, p3 - 3 kDa product, p7 - 3 kDa product).

Table 1. Examples of common APP mutations that promotes generation of $A\beta_{42}$.

APP Mutation	Amino acid change	Site of APP mutation
Swedish	Lysine > Asparagine Methionine >Leucine	670 671
English	Histidine > Arginine	677
Tottori	Aspartic acid > Asparagine	678
Taiwanese	Aspartic acid > Histidine	678
Leuven (Italian)	Glutamic acid > Lysine	682
Flemish	Alanine >Glycine	692
Arctic	Glutamic acid > Glycine	693
Italian	Glutamic acid > Lysine	693
Dutch	Glutamic acid > Glutamine	693
Iowa	Aspartic acid > Asparagine	694
Austrian	Threonine >Isoleucine	714
Iranian	Threonine > Alanine	714
German	Valine > Alanine	715
French	Valine > Methionine	715
Florida	Isoleucine > Valine	716
Indiana	Valine > Phenylalanine	717
London	Valine > Isoleucine	717

ER stress activates PERK-dependent Unfolded Protein Response signaling pathways

The ER is a network of tubules and sacks that extend in the cell cytosol. ER plays a vital role in protein synthesis, post-translational modifications and protein folding. Moreover, ER provides proper biosynthesis of phospholipids and maintains calcium homeostasis (Rutkowski & Kaufman 2004, Cooper 2000). A range of stressful, pathological conditions have a significant influence on ER function. Stress stimuli include deprivation of nutrients, changes in redox homeostasis, increased level of protein synthesis, hypoxic conditions, viral infections as well as deficiency of calcium ions in the ER lumen (Pytel et al. 2014). It has been reported that one important role of ions calcium is to support functioning of ER chaperones and maintain proper protein folding. Current estimates have suggested that Aβ peptides trigger release of calcium ions toward cell cytoplasm. ER lumenal depletion of calcium ions has a negative impact on chaperone activity as well as protein folding (Leissring et al. 2001). Disturbances in physiological functions of ER evoke ER stress, which activates PERK-dependent UPR signaling network known as a set of pro-adaptive signaling pathways, which the main aim is to restore ER homeostasis (Xu et al. 2005). Above-mentioned adaptive response involves enhanced expression of genes, which are responsible for proper protein folding within ER lumen as well as degradation of pathological proteins. Due to adverse conditions misfolded and unfolded proteins are accumulated within the ER lumen, and subsequently global protein synthesis is inhibited to reduce influx of a new, aberrant proteins into the ER lumen (Xu et al. 2005). Generally, native monomeric proteins possess α-helix confirmation, but the characteristic feature of misfolded proteins is β-sheet confirmation. It allows to conclude that aggregation of aberrant proteins within the ER lumen is the main cause of neurodegenerative diseases (Doyle et al. 2011). Paradoxically, UPR has a dual role, since if during prolonged stress conditions the pro-survival response fails apoptotic cell death ensues (Vandewynckel et al. 2013). Interestingly, UPR is implicated in the pathogenesis of numerous human disease entities such as neurodegenerative diseases, including AD, cancer and a range of inflammatory diseases like atherosclerosis, type II diabetes, renal disease, arthritis as well as inflammatory bowel disease (Brown & Naidoo 2012, Tabas & Ron 2011).

The lumen of the ER is crowded with folding enzymes and chaperones such as immunoglobulin heavy chain-binding proteins (BiP), that play a major role in all stages of protein folding (Brown & Naidoo 2012). Under normal conditions they create a specific complex with inactive ER transmembrane receptor like PERK (Vandewynckel et al. 2013), which belongs to the serine/threonine protein kinase. During aggregation of misfolded and unfolded protein within the ER lumen BiP dissociate from receptors' catalytic domains and PERK undergoes oligomerisation and trans-autophosphorylation, that trigger its rapid activation (Harding et al. 1999, Ma & Hendershot 2002). Activated PERK subsequently phosphorylates Eukaryotic Initiation Factor 2 alpha (eIF2α) at Ser51 (Doyle et al. 2011) resulting in significant attenuation of global protein translation and induction of translation of only selective mRNA such as secretase β, ATF4 and CCAAT-enhancer-binding protein homologous protein (CHOP), that may trigger cell death via apoptosis (Blais et al. 2004, Nishitoh 2012, Devi & Ohno 2014).

It has been reported that eIF2 consists of three major parts such as subunits: α, β and γ (Suragani et al. 2006). Translation initiation is strictly dependent on eIF2, since it possesses the ability to create a multiprotein complex with guanosine triphosphate (GTP) and initiator-methionyl-tRNA. Subsequently, that complex interacts with the smaller ribosomal subunit termed 40S, which results in creation of pre-initiation complex 43S, that is directly responsible for the initiation of protein translation (Kimball 1999). Above-mentioned complex binds to mRNA and moves downstream toward the initiation codon AUG. As a consequence of correct codon-anticodon pairing the 48S pre-initiation complex is formed. During that process energy is released, since GTP is hydrolyzed to GDP via GTP-ase-activating protein eIF5 (Elsby et al. 2011). Creation of a new ternary complex is closely connected with conversion of GDP to GTP, which is catalyzed by guanine nucleotide exchange factor eIF2β. Interestingly, phosphorylation at Ser51 subunit α of eIF2 by activated PERK, under ER stress conditions, triggers inhibition of global protein synthesis, since exchange of GDP to GTP is abrogated. As a result formation of a new ternary complex and subsequent protein translation is effectively inhibited (Krishnamoorthy et al. 2001).

There is abundant evidence that the level of phosphorylated eIF2α is significantly increased in AD brain tissue. Besides, currently many studies were undertaken to confirmed increased level of phosphorylated eIF2α in transgenic AD mouse models with memory impairments such as 5XFAD and APP/PS1 KI. These studies have shown that aberrant activation of PERK kinase in AD brain may represent the main mediator of eIF2α phosphorylation at Ser51 in AD brains (Duran-Aniotz et al. 2014). Likewise, recent data have suggested that increased level of phosphorylated eIF2α in tissue brain of AD patients and APP transgenic mice is accompanied with higher level of secretase β, that is responsible for the activation of Aβ production (Devi & Ohno 2014). It has been reported that monomers of Aβ become toxic for brains nervous tissue after their aggregation into oligomers, and then in higher aggregated conformations (Lorenzo & Yankner 1994, Pike et al. 1991).

Molecular mechanisms of ER stress-induced apoptosis

The crucial role of the activation of UPR signaling network is to rebalance ER homeostasis. Generally, UPR is known as a molecular mechanism by which ER copes with adverse, pathological conditions (Wagner & Moore 2011). During severe and long-termed stress conditions pro-adaptive signaling pathways of the UPR are insufficient. Persistent activation of ER transmembrane receptors and CHOP may evoke cell death via apoptosis (Szegezdi et al. 2006). The characteristic hallmark of CHOP is its expression at low concentrations during physiological conditions, but its synthesis is significantly elevated under prolonged stress conditions. Transcription of CHOP occurs when ER membrane-bound receptors, such as PERK, are in active state. Notably, PERK/eIF2α/ATF4 signaling network, induced by ER stress, triggers markedly elevated expression of CHOP, that results in apoptotic cell death (Oyadomari & Mori 2004).

There is a body of evidence suggesting that ER stress-induced apoptosis is closely associated with human disease entities including neurodegenerative diseases (Tabas & Ron 2011), but the mechanism

responsible for switching pro-adaptive signaling branches into pro-apoptotic still remains unclear (Doyle et al. 2011). High level of phosphorylated eIF2α, as a consequence of PERK activation, results in enhanced translation of ATF4. That stimulates expression of numerous genes responsible for adaptive response to adverse conditions (Schonthal 2012). On the contrary, during ER stress conditions, ATF4 as a transcription factor upregulates expression of pro-apoptotic *DNA Damage Inducible Transcript 3 (DDIT3)* gene that encodes CHOP protein (Dey et al. 2010). CHOP is involved in downregulation of the expression of the anti-apoptotic *Bcl-2* gene family and, adversely, upregulation the pro-apoptotic genes encoding proteins such as Bcl-2-like protein 11 (Bim), BH3 Interacting Domain Death Agonist (Bid), Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), p53 upregulated modulator of apoptosis (Puma) among others. Above-mentioned proteins are the members of the BH3 domain-only proteins and possess the ability to the induction of apoptosis via attenuation of biological activity of anti-apoptotic Bcl-2 proteins (McCullough et al. 2001, Shamas-Din et al. 2011).

Furthermore, one of the transcriptional target of CHOP is Growth arrest and DNA damage-inducible protein (GADD34), which may dephosphorylate eIF2α, thus lead to global translation recovery under non-physiological, stress conditions (Marciniak et al. 2004). Upon this negative feedback loop GADD34 directly combine with protein phosphatase 1 (PP1). That complex dephosphorylates eIF2α, triggers translational recovery, thus promotes ER nascent protein loads, ER stress and, as a result, apoptotic cell death (Feldman et al. 2005, Brush et al. 2003).

CHOP also significantly increases expression of *ER oxidoreductin 1α (ERO1α)* genes. Products of that genes, under ER stress conditions, promotes a hyperoxidizing environment, that evokes cell death via apoptosis (Sevier & Kaiser 2008, Simmen et al. 2010). Additionally, CHOP, upon oxidized conditions, may also activate calcium-release channel inositol-1,4,5-trisphosphate receptor 1 (IP3R1). Finally, leakage of calcium ions into the cell cytoplasm activates calcium-sensing kinase termed calcium/calmodulin-dependent protein kinase II (CaMKII). That events results in the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) subunit 2 (NOX2) and subsequent production of reactive oxygen species (ROS). That creates a positive feedback loop, since ROS generates by NADPH oxidase promotes expression of CHOP and, as a consequence, apoptotic cell death (Tabas & Ron 2011) (Fig. 2).

Conclusions

Dementia constitutes one of the primary health problem. Current data suggest that approximately 24 million people suffer from AD and 80% cases involve mutations in genes. The characteristic feature of AD is a deposition of senile plaques mainly composed of toxic form of Aβ consisting of 42 amino acids, which leads to neuronal loss and impairment of memory function in AD patients. Despite, the molecular basis of AD is not fully understood, recent study has suggested that the core of the AD disorders lies in genetic factors. Mutations in *APP* gene on chromosome 21. activate amyloidogenic pathways, where an essential role, during generation of toxic Aβ42, plays secretase β. Moreover, recent data have suggested that

disturbances on the molecular level are closely associated with ER stress an subsequent activation of PERK-dependent UPR signaling branches, that possess a dual role such as pro-adaptive and pro-apoptotic, which depends on the severity of stress conditions as well as exposure time of neurons to unfavorable factors.

Nowadays, only symptomatic treatments is available for cognitive decline in AD. Current data suggest that attenuation of PERK via its small-molecule inhibitors may prevents excessive phosphorylation of eIF2α, thus block enhanced β-amyloidogenesis through significant decline in APP cleaving in amyloidogenic pathway, what seems to be important in future therapies.

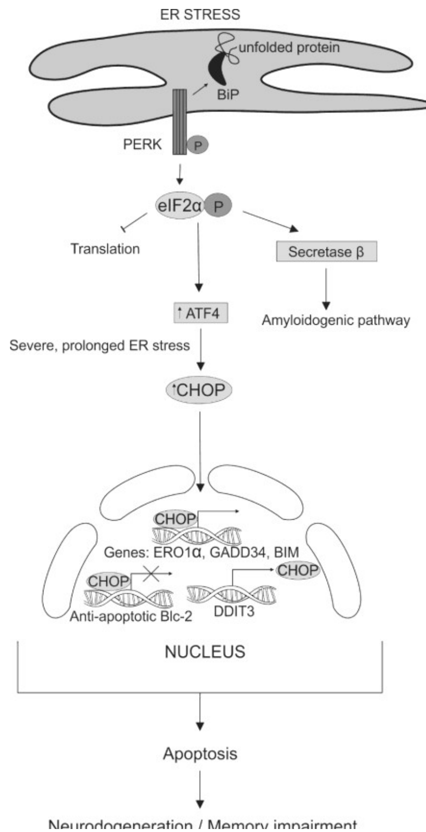

Figure 2. Mechanisms of the activation of the pro-adaptive response and CHOP-induced apoptosis under ER stress conditions (ER – Endoplasmic Reticulum, P – phosphate group, BiP - immunoglobulin heavy chain-binding proteins, PERK - protein kinase RNA-like endoplasmic reticulum kinase, eIF2α - Eukaryotic Initiation Factor 2 alpha, ATF4 - Activating Transcriptor Factor 4, CHOP - CCAAT-enhancer-binding protein homologous protein , ERO1α - ER oxidoreductin 1α, GADD34 - Growth arrest and DNA damage-inducible protein, BIM - Bcl-2-like *protein* 11, Bcl-2 - protein B-cell lymphoma 2, DDIT3 - DNA Damage Inducible Transcript 3).

Acknowledgements

This work was supported by grant PRELUDIUM no. 2015/19/N/NZ3/00055 from the National Science Centre, Poland, grant HARMONIA no. 2013/10/M/NZ1/00280 from

the National Science Centre, Poland and by grant of Medical University of Lodz no. 502-03/5-108-05/502-54-170.

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Streszczenie

Choroba Alzheimera (ang. *Alzheimer's disease*, AD) jest przewlekłą, najczęściej występującą, chorobą neurodegeneracyjną prowadzącą do nieodwracalnych zmian w strukturze, biochemii i funkcjach mózgu. Neurodegeneracja Ośrodkowego Układu Nerwowego (OUN) jest wynikiem odkładania toksycznych złogów amyloidu β (Aβ) w tkance nerwowej mózgu. Rozwój AD jest przyczyną skomplikowanych interakcji między podłożem genetycznym, a czynnikami biologicznymi, które aktywują złożone szlaki molekularne w przebiegu schorzenia. Za jedną z głównych przyczyn uważa się mutacje występujące w genie kodującym Prekursorowe białko amyloidu β (ang. *Amyloid beta Precursor Protein*, APP) zlokalizowane w pobliżu cięcia białka APP przez wysoce specyficzne sekretazy: α, β oraz γ. Generowanie toksycznej formy Aβ o długości 42-óch aminokwasów, odkładanego w tkance mózgowej jako płytki starcze, zachodzi poprzez drogę amyloidogenną, w której uczestniczą sekretazy β oraz γ.

Na podłożu molekularnym główną przyczyną rozwoju choroby AD jest akumulacja błędnie sfałdowanych lub niesfałdowanych białek w lumen Retikulum Plazmatycznego (ang. *Endoplasmic Reticulum*, ER). Skutkuje to bezpośrednim wywołaniem stresu ER, który prowadzi do aktywacji kinazy PERK, a następnie fosforylacji eukariotycznego czynnika inicjacji translacji 2 (eIF2α). W rezultacie w komórce nerwowej inhibowana jest translacja większości białek oraz dochodzi do preferencyjnej translacji wyłącznie takich białek takich jak ATF4 (ang. *Activating Transcriptor 4*) oraz, wyniku długotrwałych warunków stresowych, CHOP (ang. *CCAAT-enhancer-binding protein homologous protein*).

Nadekspresja białka CHOP prowadzi do wzmocnienia ekspresji genów kodujących: pro-apoptotyczne białka BH3 domain-only, GADD34 (ang. *DNA damage-inducible protein*, GADD34 oraz białko o aktywności oksydoreduktazy ER (ang. *ER oxidoreductin 1α*, ERO1α). W warunkach wysokiego stężenia białka CHOP zostaje osłabiona ekspresja genów kodujących anty-apoptotyczne białka Bcl-2. W rezultacie masa tkanki nerwowej mózgu ulega znaczącemu obniżeniu w wyniku postępującego procesu neurodegeneracji na drodze apoptotycznej śmierć komórkowej w przebiegu AD.

Folia Biologica et Oecologica 12: 60 (2016)
Acta Universitatis Lodziensis

Erratum to: The possibilities of using data on national populations of fungal species to determine the Red List Category (based on the IUCN Criteria)

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Erratum to: Kędra K. (2014) The possibilities of using data on national populations of fungal species to determine the Red List Category (based on the IUCN Criteria). Volume 10, pages: 122–129 (DOI: 10.2478/fobio-2014-0015):

All references were changed as follows:

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DOI: 10.1515/fobio-2015-0006