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REVIEW

The Tumor Cell and Telomerase

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Abstract—Imbalanced activity of the mechanism that controls cell division is a prerequisite for malignant transformation of a normal cell. The present review considers this multi-step mechanism, which is usually called the G_1–S checkpoint. Besides, tumor cells are characterized by the presence of telomerase, an enzyme responsible for restoration of chromosome ends after replication and thus providing for unlimited cell division. The main point of the present article is to find out whether the activation of telomerase is controlled by the G_1–S checkpoint or does not depend on it. The principal components of the G_1–S checkpoint, such as cyclin-dependent kinases, retinoblastoma and E2F proteins, control the activity of telomerase. In their turn they accumulate and transmit signals from various sources inside and outside the cell. Thus, various changes in tumor cells can activate telomerase through the G_1–S checkpoint. Such are the suggested effects on telomerase of Myc, p53, Waf1, protein kinases B and C, Wnt5A, TGFβ, WT1, and estrogens. However, Myc, p53, WT1, estrogens, protein kinases B and C, and TGFβ can also directly influence telomerase independently of the G_1–S checkpoint mechanism. Moreover, in 30% of human tumors the gene of the key subunit of telomerase (hTERT) is amplified, possibly due to chromosomal rearrangements unassociated with the activity of the G_1–S checkpoint. Thus, telomerase seems to be activated not by a single agent but due to combined action of various factors, both with involvement of the G_1–S checkpoint mechanism and independently of it.

Key words: telomerase, cell cycle, malignant transformation, cancer

Despite significant progress in understanding molecular events that underlie malignant transformation of a normal cell, medicine is still far from victory over cancer. A great variety of causes resulting in development of tumors prevents the elaboration of effective approaches for treatment of tumors. On the molecular level this variety is observed even in tumors of the same histological type [1]. Activation of the enzyme telomerase is the only common change observed in the majority of malignancies. This fact was discovered in 1994 [2] and caused the avalanche-like growth of interest in telomerase as a promising universal target for antitumor drugs.

By now, there are thousands of studies on telomerase. These works are mainly searches for telomerase inhibitors and studies on its activity in tumors and biological regulation of this enzyme. This review is an attempt to describe the most typical features of the tumor cell and to find a possible regulatory connection between telomerase and other factors that determine malignant transformation.

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1. CENTRAL ROLE OF THE G_1–S CHECKPOINT IN THE ORIGIN OF TUMORS

The ability for uncontrolled division that is specific for tumor cells suggests disorders in the regulatory mechanism of the cell cycle. Consider in general the structure and principle of action of this mechanism (Fig. 1).

Genes required for the replication are directly activated by the protein E2F. The effect of E2F is controlled by the retinoblastoma-associated protein (Rb). Rb exists in two forms, phosphorylated and unphosphorylated. Unphosphorylated Rb binds to E2F and inhibits its activity. In its turn, Rb is controlled by cyclin-dependent kinases (Cdk). The activity of Cdk is stimulated by cyclins and inhibited by proteins INK4, Kip1, and WAF1. The protein Myc seems to prevent the effect of Cdk inhibitors and also to activate the E2F gene [3-6]. Irradiation or chemical carcinogens activate the protein p53, which through WAF1 blocks the initiation of replication until DNA damage has been removed by reparative enzymes. Thus, p53 prevents appearance of mutations caused by inclusion of incorrect nucleotides during the replication

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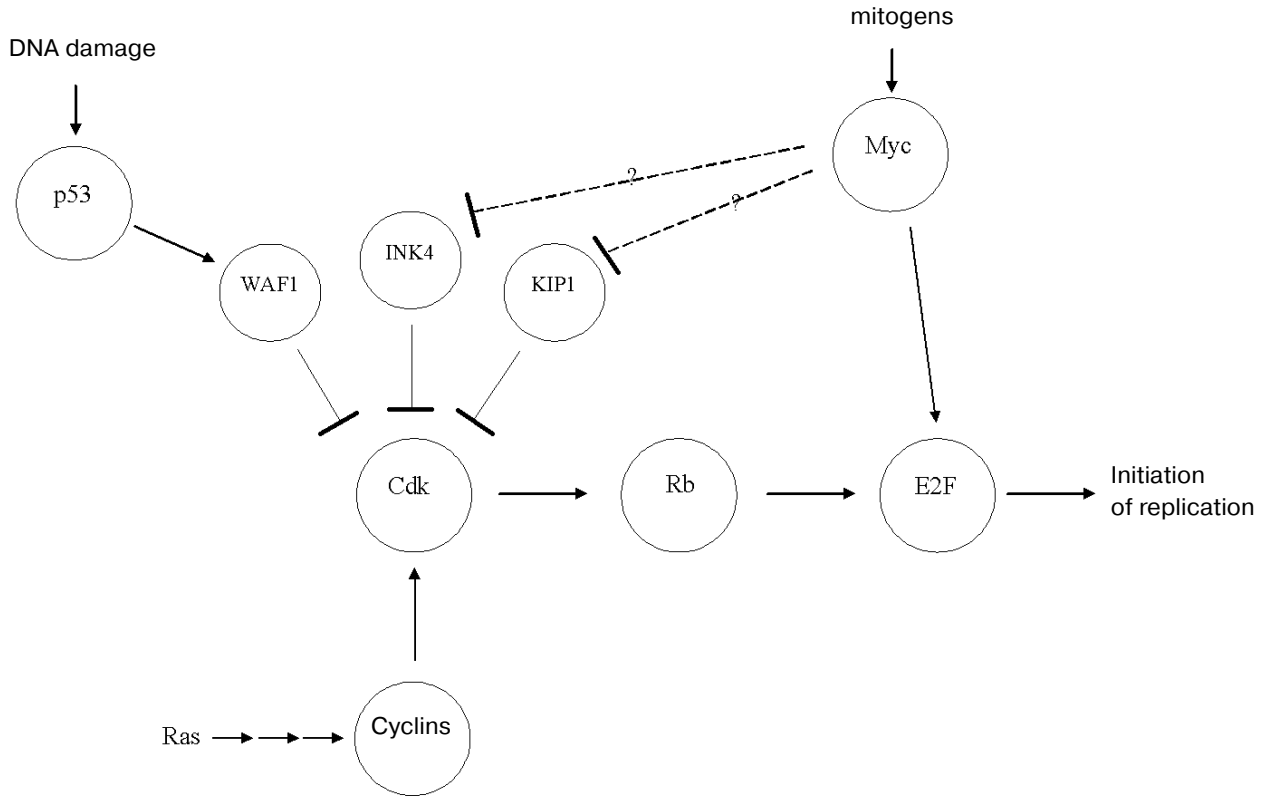

Fig. 1. Main steps of the system of cell division control in humans and other vertebrates.

of impaired DNA regions. Preventing mutagenesis at large, p53 also decreases the risk of mutations that promote the malignant transformation of a normal cell [7].

Inadequate functions of this mechanism in tumor cells can be caused either by disorders in just this mechanism or by transmission of erroneous signals from outside. For instance, the function of the Rb protein is disturbed in leukemias [3], small- and non-small-cell lung cancer [6], and hepatocarcinoma [8]. The content of cyclin E is increased in 40% of cases of non-small-cell lung cancer [6]. A constitutive activation of a signal protein Ras in several tumors [9, 10] makes the control system of cell division transmit stimulating signals, which cause the unlimited cell division. No active p53 protein has been found in about 50% of human tumors [7].

Though simplified, the scheme in Fig. 1 presents a universal principle of regulation of cell division in humans. Figure 1 shows only the central part, the core of the mechanism that accepts information transmitted via regulatory chains from various sources inside and outside the cell and converts this information into a signal initiating replication. Malignant transformation is unlikely to exist without the involvement of the G_1–S checkpoint mechanism, but it can also involve other events unrelated to this mechanism. Thus, at a certain stage of tumor development its cells acquire abilities to produce substances that regulate angiogenesis [11], destroy tissues surrounding the tumor [12], penetrate through vascular walls, and spread over the body through the blood flow [13]. Some mutations which increase the genome instability and, similarly to mutations in the p53 gene, increase the risk of malignant transformation are also unrelated with changes in the G_1–S checkpoint. Thus, colon tumors are characterized by deficiencies in genes encoding components of the mismatch repair system that eliminates errors of the replicative DNA polymerase [14].

In addition, the body protects itself against tumors by elimination of malignant cells by apoptosis. However, tumor cells are sometimes resistant to apoptosis. This resistance is caused by various mechanisms [15, 16]. In some tumors there is a high level of expression of the bcl-2 protein which inhibits self-elimination of cells at the mitochondrial step of apoptosis [17, 18]; in other cases the function of intracellular components, such as ATM [3] or ARF [8] proteins, which are functionally associated with p53, is disturbed. The loss of p53 function results not only in the loss of ability to stop the cell cycle for a while but also in resistance to apoptosis, because this protein also induces apoptosis in the presence of carcinogens or excess mitogenic signals [19]. The G_1–S checkpoint mechanism that is central in carcinogenesis is associated with apoptosis. Excess stimulation of this regulatory sys-

tem by signals from the environment or from intracellular disorders causes programmed cell death mediated through ARF and p53 [8]. However, in some cases the resistance of tumor cells to apoptosis can be unassociated with activity of the G_1–S checkpoint mechanism.

2. TELOMERE AND TELOMERASE

Leaving for the moment the cancer problem, we consider some specific features of chromosome replication. During the replication, the lagging chain of DNA is known to be initially produced as discrete Okazaki fragments each of which results from elongation of an RNA primer (Fig. 2). In the next stage of synthesis of the lagging chain, RNA primers are removed by endonuclease and the empty space is filled in with DNA. DNA polymerase responsible for this needs a primer, and this role is played by the 3′-end of the nearest Okazaki fragment. Ribonuclease also destroys primers located at the chromosome edge (shown by dotted line in Fig. 2). However, these RNA primers cannot be replaced by DNA because of absence of Okazaki primer for DNA polymerase. As a result, a void is produced instead of terminal RNA primers. Thus, each replication cycle is accompanied by an irreversible loss of a small region at the chromosome end. This so-called terminal under-replication has been shown experimentally. It results in progressive shortening of chromosomes in the course of cell divisions [20, 21]. The shortening of chromosomes finally causes cell death.

On the ends of human chromosomes there is a multiply repeated 5′-TTAGGG sequence. The region containing these repeats is called a *telomere*. With each cell division the telomere is shortened by 50-200 nucleotides [22]. In fact, it plays the role of a buffer zone that ensures a certain number of cell divisions without the loss of vitally important regions located near the chromosome ends [23, 24].

The ability of tumor cells for unlimited division requires the problem of terminal replication to be solved. In fact, tumor cells contain the enzyme *telomerase* that prevents the shortening of telomeres. Telomerase elongates the 3′-end of chromosome by addition of TTAGGG links to it. The other chain of DNA is completed in the ordinary way with involvement of primase, DNA polymerase, and DNA ligase [25]. With some rare exceptions, healthy human somatic cells have no telomerase activity. In contrast, about 90% of tumors have active telomerase [2, 21].

3. G_1–S CHECKPOINT AND ACTIVATION OF TELOMERASE

Thus, elucidation of the cause–effect relationship of events resulting in malignant transformation should include the detection of factors responsible for activation of telomerase in tumors. The present review considers a possible involvement of the G_1–S checkpoint regulatory system in the activation of telomerase. In this connection, two opposing ideas should be considered:

1) telomerase is activated by signals from the G_1–S checkpoint;

2) telomerase is activated independently of the G_1–S checkpoint functioning.

The second idea is in agreement with the generally accepted concept on malignant transformation of a normal cell as a result of a stepwise accumulation of mutations in various genes associated with division, growth, and (in metastasizing) spreading over the body. Thus, the combined activation of telomerase and disorders in the G_1–S checkpoint mechanism can be a result of two independent events (usually of mutations). This opinion is supported, in particular, by the finding of increased copy number of the hTERT gene encoding the catalytic subunit of telomerase in 30% of human tumors [26].

4. CURRENT STATE OF THE PROBLEM OF REGULATION AND CAUSES OF TELOMERASE ACTIVATION IN HUMAN TUMOR CELLS

Telomerase is isolated from tissues as a ribonucleoprotein consisting of three subunits: RNA (hTER), the hTERT polypeptide, and another polypeptide hTEP1. hTER contains a region of 11 nucleotides complementary to one of the telomere chains which plays the role of RNA template for elongation of this chain. The hTERT polypeptide is a reverse transcriptase that synthesizes the DNA chain on the hTER template [27, 28]. The hTEP1 protein is likely to have a regulatory function (see below). Active telomerase can be constructed *in vitro* without TEP1 by binding hTER and hTERT [29-31]. Both hTER and hTERT are undeniably required for telomerase functioning. Consequently, studies on regulatory mechanisms of expression and activation of these components must be central in studies designed to answer why telomerase is activated in tumor cells.

Neither hTER, nor hTERT are usually found in somatic organs of healthy adults [32, 33]. However, in cell cultures from these organs only hTERT is repressed, whereas the expression of hTER and TEP1 seems to be constitutive [32-35]. The constitutive expression of hTER in cell cultures makes difficult studies on the regulation of this component of telomerase. This, in particular, can be the reason for the extremely small number of studies concerning hTER regulation [33]. On the contrary, regulation of the hTERT protein has been studied very intensively and now many data are available for discussion. Therefore, nearly all data considered in this review refer to hTERT.

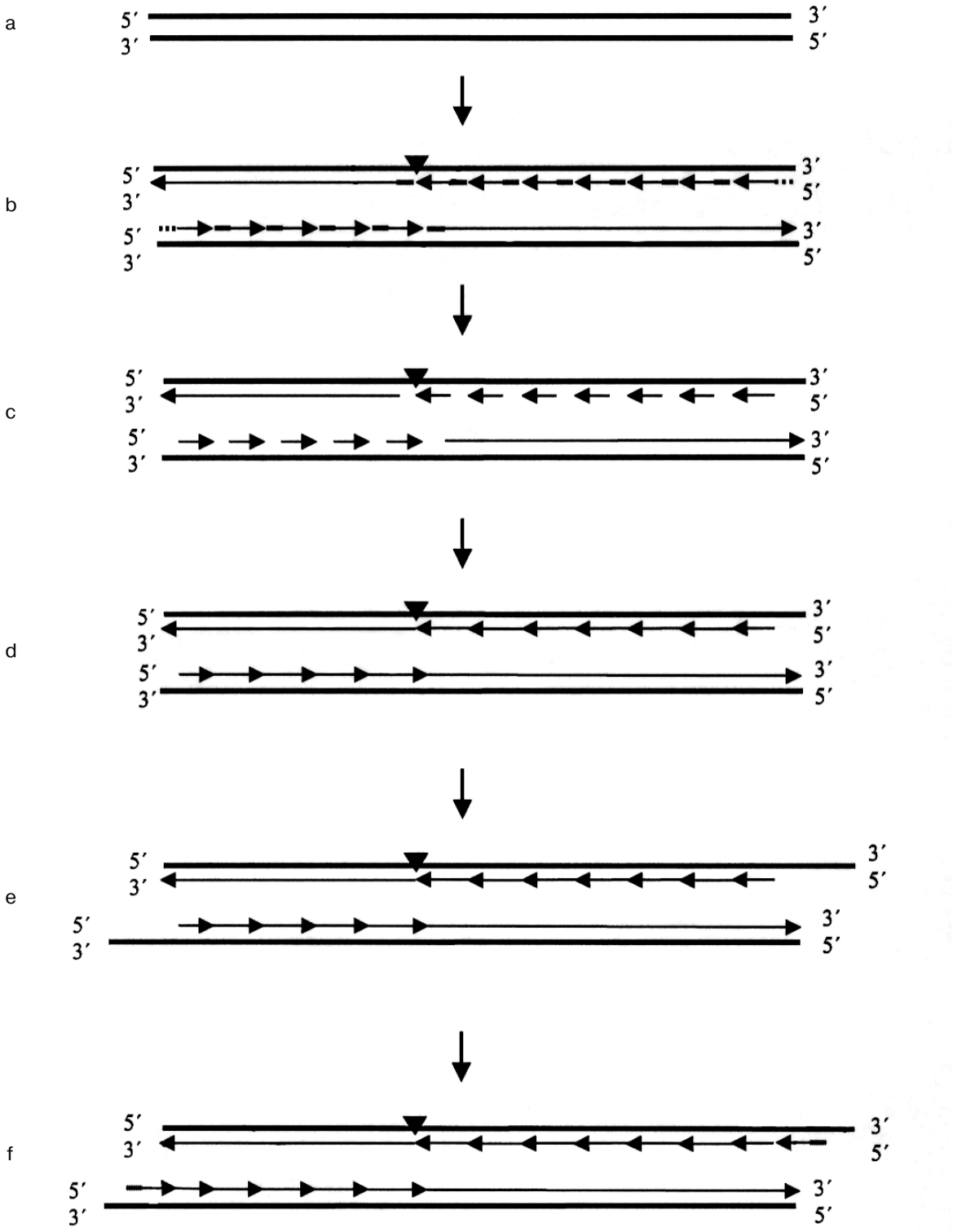

Fig. 2. Problem of terminal under-replication and the function of telomerase: a) parental DNA; b) DNA during replication (the triangle designates the replication origin, arrows — Okazaki fragments, dotted lines and bold dashes — primers of Okazaki fragments and of leading strands; dotted lines show the end primers); c) DNA during replication after removal of RNA primers; d) DNA in the terminal stage of replication when voids between Okazaki fragments are filled in with DNA and bound by ligase. Voids in places of the removed end primers cannot be filled in with DNA, and this results in shortening of the daughter chains; e) telomerase elongates the protruding 3′-end of DNA; f) primase produces a new RNA primer and DNA polymerase fills in the DNA region that included the removed terminal primer.

It should be kept in mind that hTERT can be regulated on several levels:

1) activation or repression of the hTERT gene transcription;

2) control of the transcript splicing;

3) posttranslational modification of the hTERT protein.

Normal cells, as a rule, contain neither mRNA nor the protein product of the hTERT gene [36, 37]. The hTERT promoter is active in immortal tumor cell lines, such as HeLa, and is inactive in cultures of normal cells [38, 39]. Consequently, activation of telomerase can be partially associated with activation of the hTERT gene promoter. Splicing of the primary transcript of the hTERT gene is also under regulation because it is carried out in several ways, and only one of them results in production of normal mRNA [40, 41]. And finally, the hTERT polypeptide can be modified by phosphorylation with different protein kinases [42, 43].

A review of data on the role of the G_1-S checkpoint in telomerase regulation is presented below. We begin it with consideration of the central components of the G_1-S mechanism—cyclin-dependent kinases, Rb and E2F proteins. As shown in the scheme in Fig. 1, these proteins must accept and transmit all signals from other elements of the G_1–S checkpoint. Afterwards, it is reasonable to consider involvement in the regulation of telomerase of the Myc, p53, and WAF1 proteins which are well-known regulators of the cell cycle and apoptosis and whose functions are changed or disturbed in many tumors. Later we shall consider the role of protein kinases B and C and of other signaling mechanisms associated with the G_1–S checkpoint and involved in regulation of telomerase activity (Fig. 3).

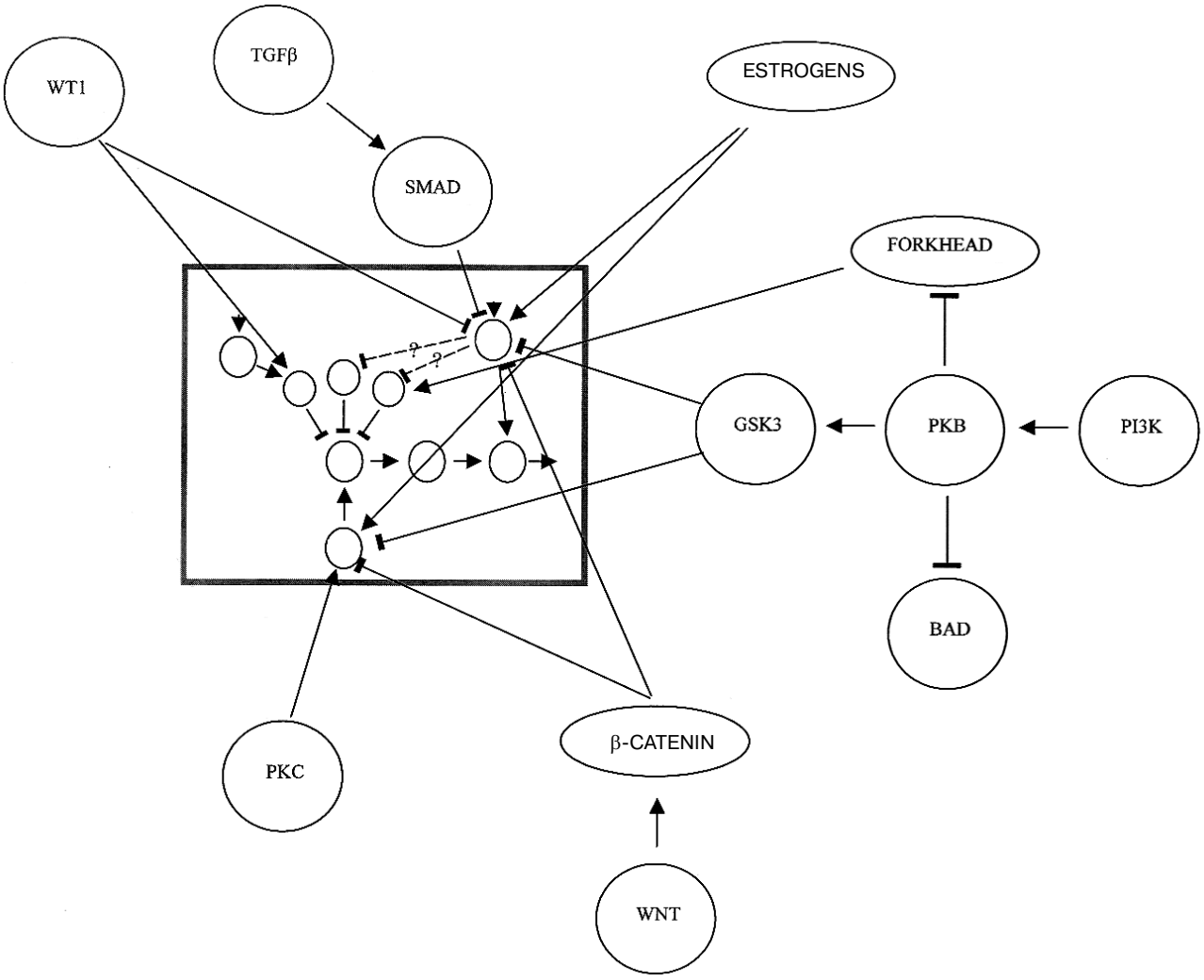

Fig. 3. Transmission pathways of mitogenic signals from telomerase regulators to the G_1–S checkpoint. The G_1–S checkpoint mechanism presented in Fig. 1 is shown inside the frame in the center of the figure. In the periphery the regulators of telomerase considered in Section 4 are presented as circles and ellipses. The lines show their possible interactions with the G_1–S checkpoint components. Arrows show activation and transverse dashes show inhibition.

E2F, Rb, and cyclin-dependent kinases. Crowe and Nguyen [44] showed that an artificial increase in the intracellular concentration of the unphosphorylated Rb protein decreases the activity of telomerase in human carcinoma cells. The concurrent increase in concentrations of two proteins, Rb and E2F, abolished the inhibitory effect of Rb on telomerase and returned its activity to the control level [44]. The inhibitory effect of Rb also disappeared in the case of concurrent increase in the intracellular concentrations of Rb and cyclin-dependent kinases, Rb and cyclin. These results agree with the scheme presented in Fig. 1 and suggest that E2F can activate telomerase.

However, the same authors in another work on the same cell line clearly demonstrated that E2F binds to the hTERT gene promoter and suppresses the activity of telomerase as a repressor of its gene [45]. Obviously, these results do not agree with the data of [44] if they are interpreted on the basis of the mechanism presented in Fig. 1. The authors do not comment on this contradiction. Anyhow, it is important that these experiments allow for a general conclusion that telomerase is controlled by the key elements of the G_1–S checkpoint.

Myc. The DNA region adjacent to the 5′-end of the hTERT gene includes several sites which are necessary for attachment of Myc (E boxes) and actually bind this protein *in vitro* [38]. Myc and the telomerase activity are closely associated in cell cultures of various origins [38, 46]. An artificial increase in the intracellular concentration of Myc stimulated transcription directed by the hTERT gene promoter [38, 47-50]. In contrast, selective suppression of Myc synthesis by introduction into cells of the anti-sense oligonucleotide complementary to mRNA of Myc decreased the activity of the hTERT promoter or [38].

p53. Sp1 is another activator of the hTERT promoter. In cell cultures, this protein binds to p53 with high specificity. The protein–protein interaction of p53 and Sp1 prevents the Sp1 functions on the hTERT gene. Thus, p53 suppresses the hTERT expression and inhibits telomerase [51-53].

Moreover, in lysates of cell nuclei p53 has been independently found to inhibit the telomerase activity by binding to the hTEP1 protein, which is the third component of the enzyme [54].

Thus, another side of the antitumor effect of p53 is displayed by its anti-telomerase effect.

WAF1. Because p53 and WAF1 are elements of the same signaling chain (see Section 1), a question arises whether WAF1 is an intermediary between p53 and telomerase. This question was thoroughly investigated in above-mentioned works [51-53] and was answered in the negative. However, under some conditions and possibly without relation to p53, WAF1 can influence telomerase. Transfection of human tumor cells with plasmid that contained the WAF1 gene inhibited growth concurrently with decrease in telomerase activity [55].

Protein kinases B and C. One of the main pathways of transmission of proliferative signals from the environment into the cell nucleus depends on the coupled function of *phosphatidylinositol-3-phosphate kinase* (PI3K) and *phosphatidylinositol-3-phosphate-dependent kinase* (PDK). Activation of membrane receptors by growth factors results in fast local accumulation of phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) in the membrane. This lipid is synthesized with involvement of PI3K [56, 57]. $PI(3,4,5)P_3$ is required for activation of PDK [56]. Moreover, $PI(3,4,5)P_3$ seems to act as an anchor binding PDK and its substrates to the membrane [58]. PDK phosphorylates other protein kinases and thus activates them. Those, in particular, include serine-threonine protein kinases B and C (PKB and PKC, respectively).

PKB is responsible for three phosphorylation reactions, each of which gives impetus to malignant transformation [58]:

1) acquisition of phosphate group by proteins of the *Forkhead* family prevents their translocation from the cytoplasm into the nucleus. These proteins regulate transcription and, in particular, activate the KIP1 gene;

2) phosphorylation of the Bad protein inhibits its functioning during apoptosis and makes the cells resistant to apoptosis;

3) phosphorylation of the GSK3 protein results in stabilization and activation of Myc and cyclin D.

Experiments described in [59] and [60] suggest that PKB can also directly phosphorylate hTERT and activate telomerase.

The term “protein kinase C” refers to a group of enzymes with similar structure. Upon activation with diacylglycerol phosphorylate they phosphorylate various protein substrates on serine or threonine [61, 62]. Protein kinase C is responsible for transmission of different signals inside the cell. In particular, it is involved in the regulation of cell division and apoptosis. In many cases, the mechanism of this regulation is complicated, variable, and unclear. However, protein kinase C is known to be involved in transmission of signals from growth factors to cyclin D and the G_1–S checkpoint [56, 61].

The activity of some forms of protein kinase C is often increased in tumor cells [42]. Protein kinase C is chosen as a target for some antitumor drugs [61].

Protein kinase C phosphorylates hTERT and hTEP1 *in vitro* [63]. In cell cultures of mammary gland cancer and nasopharynx carcinoma and also in culture of blood mononuclear cells protein kinase C seems to be an enzyme which mediates phosphorylation of protein subunits hTERT and hTEP1 of telomerase, and this function of protein kinase C is needed for the activity of telomerase [63-66].

Wnt5A. Transcription of some genes with various functions is controlled by the protein β-catenin [67]. β-Catenin itself is controlled by signaling proteins Wnt that

are located in the intercellular space and realize their effect through a receptor and a chain of mediators. Combined with intermediate links of the signaling chain and additional components influencing the stability and specificity of β-catenin, this protein forms a cascade called the *Wnt/β-catenin signaling pathway* [67]. Mutations in the genes encoding elements of this signaling cascade are specific for a number of tumors [8, 67].

The cyclin D_1 and Myc genes are important targets for Wnt and β-catenin [8].

Expression of the wnt5a gene in culture of human kidney carcinoma correlated with decrease in the telomerase activity in these cells [68].

Autocrine transforming factor β (TGFβ). The cytokine TGFβ, its membrane receptors, and proteins Smad present another main pathway of signal transmission from the extracellular medium into the cell nucleus. These signals change activities of many genes responsible for various functions [69]. The binding of TGFβ to receptors leads to phosphorylation of Smad, which afterwards are translocated into the nucleus where they produce complexes with protein factors responsible for regulation of gene transcription. Mutations in the genes of components of this system are found in different tumors but are especially specific for tumors of gastrointestinal tract organs [69].

Smad, as antagonists of Myc in the regulation of the INK4b gene, inhibit cell division. Myc suppresses expression of this gene (see Section 1) and Smad activates it [70, 71].

With two cell lines (colon carcinoma HCT116 and breast carcinoma MCF-7) TGFβ was also shown to control the activity of telomerase and the level of hTERT mRNA [72]. Moreover, using a vector with the promotor–regulatory region of the hTERT gene combined with the luciferase gene the authors have also shown that this control is realized via changes in activity of the hTERT promotor.

As a regulator of the G_1–S checkpoint, TGFβ usually slows downs cell division, arresting the cells at the G_1 stage [73]. However, Yang et al. [72] did not observe this delay in cultures with telomerase inhibited by TGFβ. Evidently, under these conditions changes in the activity of telomerase and in expression of hTERT were not mediated through the G_1–S checkpoint.

WT1. The WT1 gene encodes a number of proteins produced by alternative splicing and involved in development and differentiation of the urogenital system. Mutations in this gene are often observed in Wilms' tumor (a kind of malignant nephroblastoma) [74].

A regulatory link of WT1 with the G_1–S checkpoint has been found. In particular, it is displayed by the WT1-caused decrease in expression of the c-Myc gene [75] and increase in expression of the WAF1 gene [76]. However, it is unclear whether these effects are significant for the antitumor effect of WT1.

In human kidney cell culture a product of the WT1 gene called WT1(-KTS) binds to the promotor–regulatory region of the hTERT gene and inhibits transcription [39].

Estrogens. In mammary gland cancer estrogens are the main factor stimulating division of malignant cells [77, 78]. Estrogens penetrate into target cells, produce complexes with the protein receptor, and activate or suppress transcription of certain genes. In particular, estrogens stimulate expression of the cyclin D_1 and c-Myc genes [79].

Estrogens also directly activate the hTERT gene [80]. In normal epithelial cells of ovary, which lack telomerase, the estrogen–receptor complex binds to the promotor region of this gene and increases the transcription, and this results in induction of telomerase activity.

CONCLUSIONS

Thus, let us try to answer the question posed in Section 3 of this review on the ground of data presented in Section 4. What is the cause of the telomerase activation in tumors? Is it due to inadequate functions of the G_1–S checkpoint mechanism or independent of it?

First, telomerase (or the hTERT gene) seems to be controlled by key components of the G_1–S checkpoint, such as cyclin-dependent kinases and Rb and E2F proteins. Other factors significant for malignant transformation of the cell and for regulation of telomerase can, at least theoretically, also influence telomerase via the pathway that includes Rb and E2F because each of these factors has a regulatory connection with the G_1–S checkpoint (Fig. 3). However, some of these factors can influence telomerase bypassing this pathway and directly affecting the hTERT gene promotor (Myc, WT1, estrogens), protein regulators of this gene (p53), or hTERT and TEP1 polypeptides (Akt and p53).

Obviously, telomerase is not activated by a single push button. There are several modes and levels of regulation of telomerase. Different modes of regulation can occur under various conditions in different tissues.

Activation of telomerase is one of the key events in transformation of a normal cell to a malignant one. Understanding how this event is involved in the whole picture of the tumor cell activity is needed for an integral concept of the nature of malignant transformation and also for development of approaches for elaboration of antitumor drugs capable of inhibiting the telomerase activity in the cell.

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