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Induction of intrachromosomal homologous recombination in human cells by raltitrexed, an inhibitor of thymidylate synthase

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ABSTRACT

Thymidylate deprivation brings about "thymineless death" in prokaryotes and eukaryotes. Although the precise mechanism for thymineless death has remained elusive, inhibition of the enzyme thymidylate synthase (TS), which catalyzes the *de novo* synthesis of TMP, has served for many years as a basis for chemotherapeutic strategies. Numerous studies have identified a variety of cellular responses to thymidylate deprivation, including disruption of DNA replication and induction of DNA breaks. Since stalled or collapsed replication forks and strand breaks are generally viewed as being recombinogenic, it is not surprising that a link has been demonstrated between recombination induction and thymidylate deprivation in bacteria and lower eukaryotes. A similar connection between recombination and TS inhibition has been suggested by studies done in mammalian cells, but the relationship between recombination and TS inhibition in mammalian cells had not been demonstrated rigorously. To gain insight into the mechanism of thymineless death in mammalian cells, in this work we undertook a direct investigation of recombination in human cells treated with raltitrexed (RTX), a folate analog that is a specific inhibitor of TS. Using a model system to study intrachromosomal homologous recombination in cultured fibroblasts, we provide definitive evidence that treatment with RTX can stimulate accurate recombination events in human cells. Gene conversions not associated with crossovers were specifically enhanced several-fold by RTX. Additional experiments demonstrated that recombination events provoked by a double-strand break (DSB) were not impacted by treatment with RTX, nor was error-prone DSB repair via nonhomologous end-joining. Our work provides evidence that thymineless death in human cells is not mediated by corruption of DSB repair processes and suggests that an increase in chromosomal recombination may be an important element of cellular responses leading to thymineless death.

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1. Introduction

The enzyme thymidylate synthase (TS) catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to produce TMP, which is required for DNA replication in proliferating cells. The reaction catalyzed by TS represents the sole pathway for *de novo* TMP synthesis, and targeted inhibition of this pathway has served for many years as a common strategy in chemotherapeutic approaches. For example, the antimetabolite 5-fluorouracil (5-FU) has been put to use for many years in the treatment of a variety of cancers [1–3]. 5-FU can be metabolized to FdUMP which inhibits TS by forming a ternary complex with TS and 5,10-methylenetetrahydrofolate. 5-FU is often administered in combination with leucovorin which is metabolized to 5,10-methylenetetrahydrofolate. In turn, the formation of the ternary complex among FdUMP, TS, and 5,10-methylenetetrahydrofolate is enhanced and the anti-cancer action of 5-FU is potentiated. Although inhibition of TS is undoubtedly a major means by which 5-FU exerts its anti-cancer effects, some metabolites of 5-FU (namely, FUTP and FdUTP) do not target TS but, rather, readily incorporate into RNA and DNA. Incorporation of fluoropyrimidines into nucleic acids engenders additional cellular effects that make important contributions to the cytotoxicity of 5-FU [1,2]. In addition to fluoropyrimidine-based TS inhibitors, folate analogs have also been put to use as TS inhibitors in chemotherapeutic approaches. Raltitrexed (RTX) is an example of a folate analog that was rationally designed as a specific inhibitor of TS [3–6]. Unlike fluoropyrimidines, RTX apparently acts exclusively through TS inhibition. The use of RTX in the treatment of colorectal cancer has been approved in Europe, Canada, Australia and Japan [7,8].

Interestingly, and perhaps surprisingly, despite the fact that inhibition of TS has served for many years in chemotherapeutic strategies, the mechanism by which "thymineless stress" ultimately leads to "thymineless death" has yet to be fully elucidated. Several documented effects of TS inhibition, alone or in combination, likely contribute to cellular demise. One notable consequence of TS inhibition is the generation of nucleotide pool imbalance, which may disrupt DNA synthesis and DNA repair and lead to lethal DNA lesions [1,9–12]. Another consequence of TS inhibition that has received much attention involves the intracellular accumulation of dUMP due to the blockage of its conversion to TMP. Accumulated dUMP can be converted to dUTP which, in combination with the depletion of TTP pools, may result consequently in the misincorporation of uracil into DNA [1,3,9,12,13]. Subsequent action of base excision repair to remove uracil from DNA has been hypothesized to result in futile cycles of repair since, under conditions of thymidylate deprivation, uracil will continue to be misincorporated into DNA during repair. The hypothesized futile cycling in turn leads to DNA strand breakage which may lead directly or indirectly to cell death [1,3,9,12,13]. It must be noted that the role that misincorporation and/or excision of uracil plays in the induction of cell death through TS inhibition remains controversial as there have been a number of reports suggesting that uracil incorporation and excision is not required for thymineless death [14–18]. It is, however, well established that thymineless stress induces single- and double-strand breaks (DSBs) in chromosomal DNA [9,12,14,18–24]. Whether such DNA strand breakage is an early event that plays a causal role in thymineless death, or a late event occurring after apoptosis has already been initiated, is an issue that has proven problematic to resolve.

One interesting potential impact of TS inhibition that has received some attention in the literature is the possibility that thymidylate starvation induces homologous recombination (HR) among chromosomal sequences. Since TS inhibition leads to DNA breakage and interferes with DNA synthesis, it seems plausible to suspect that HR may concomitantly be induced because DNA breaks and replication blockage are generally viewed as being recombinogenic [25]. Potentially high levels of HR could, in turn, engender an instability that may directly or indirectly contribute to cell death or lead to genetic abnormalities following recovery from treatment with TS inhibitors. A link between HR and thymidylate starvation has been confirmed in prokaryotes and lower eukaryotes [9,26–33]. A similar link has been suggested, but not demonstrated definitively, in higher eukaryotes [14,19,34–36].

In order to better understand the relationship between HR and TS inhibition in human cells, and to gain further insight into the mechanism of thymineless death, we initiated a study of the effect of TS inhibition on intrachromosomal HR. In this paper, we describe the development of a model system using cultured human fibroblasts to attack this issue directly. Our system involves the use of novel integrated DNA substrates that enable the study of both spontaneous HR as well as HR provoked by induction of a genomic DSB. We report that treatment of human cells with the TS inhibitor RTX can increase the rate of spontaneous intrachromosomal gene conversion several-fold. In contrast, RTX treatment had no apparent effect on either DSB-induced HR or repair of genomic DSBs by error-prone nonhomologous end-joining (NHEJ). Our results suggest that a global increase in recombination activity may be an integral component of cellular responses leading to thymineless death.

2. Materials and methods

2.1. Cell culture

Normal human fibroblast cell line GM00637 (immortalized by SV40) was obtained from the NIGMS. Cells were cultured in alpha-modified minimum essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Recombination and repair substrates

Plasmids pLB4 and pTNeo99-7 (Fig. 1) were described previously [37,38]. Substrate pLB4 was derived from pTNeo99-7 by inserting a 2.5 kb HindIII fragment containing a complete functional herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene into the unique HindIII site on pTNeo99-7. This inserted "donor" fragment on pLB4 shares about 1.7 kb of homology with the *tk* portion of the fusion gene. Due to several scattered mismatches, the donor on pLB4 displays about 1%